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(Regn. No. 28 OF 2007)

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Floating Drug Delivery Systems: A Review

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Abstract

The purpose of writing this review on floating drug delivery systems (FDDS) was to compile the recent literature with special focus on the principal mechanism of floatation to achieve gastric retention. The scientific and patent literature shows increased interest in academics and industrial research groups regarding the novel dosage forms that can be retained in the stomach for prolonged and predictable period of time. One of the most feasible approaches for achieving a prolonged and predictable dug delivery profiles in the gastrointestinal tract is to control the gastric residence time, using gastroretentive dosage forms that will provide us with new and important therapeutic options. From the formulation and technological point of view, the floating drug delivery system is considerably easy and logical approach. The developments of FDDS are including the physiological and formulation variables affecting gastric retention, approaches to design single-unit and multiple-unit floating systems, advantages, formulation variables and their classification are covered in detail. This review also summarizes the in vitro techniques, *in vivo* studies to evaluate the performance and application of floating systems. These systems are useful to several problems encountered during the development of a pharmaceutical dosage form. An attempt has been made in this review article to introduce the readers to the current technological developments in floating drug delivery

Keywords: Gastric residence time (GRT), floating drug delivery systems (FDDS), single unit, multiple units, evaluation *in vitro* and *in vivo*

Introduction

Despite tremendous advancements in drug delivery the oral route remains the preferred route of administration of therapeutic agents because of low cost of therapy and ease of administration leading to high levels of patient compliance. But the issue of poor bioavailability (BA) of orally administered drugs is still a challenging one, though extensive advancements in drug discovery process are made (1).

Conventional oral dosage forms provide a specific drug concentration in systemic circulation without offering any control over drug delivery. CRDFs or controlled release drug delivery systems (CRDDS) provide drug release at a predetermined, predictable and controlled rate. The de novo design of an oral controlled drug delivery system (DDS) should be primarily aimed at achieving more predictable and increased bioavailability (BA) of drugs.

A major constraint in oral CRDD is that not all drug candidates are absorbed uniformly throughout the gastrointestinal tract. Some drugs are absorbed uniformly throughout the gastrointestinal tract. Some drugs are absorbed in a particular portion of gastrointestinal tract only or are absorbed to a different extent in various segments of gastrointestinal tract. Such drugs are

said to have an "absorption window". Thus, only the drug released in the region preceding and in close vicinity to the absorption window is available for absorption. After crossing the absorption window, the released drug goes to waste with negligible or no absorption. This phenomenon drastically decreases the time available for drug absorption and limits the success of delivery system. These considerations have led to the development of oral CRDFs possessing gastric retention capabilities (2).

One of the most feasible approaches for achieving a prolonged and predictable drug delivery profiles in gastrointestinal tract is to control the gastric residence time (GRT) using gastroretentive dosage forms (GRDFs) that offer a new and better option for drug therapy.

Dosage forms that can be retained in stomach are called gastroretentive drug delivery systems (GRDDS)[3]. GRDDS can improve the controlled delivery of drugs that have an absorption window by continuously releasing the drug for a prolonged period of time before it reaches its absorption site thus ensuring its optimal bioavailability.

Gastric emptying of dosage forms is an extremely variable process and ability to prolong and control the emptying time is a valuable asset for dosage forms, which reside in the stomach for a longer period of time than conventional dosage forms. Several difficulties are faced in designing controlled release systems for better absorption and enhanced bioavailability. One of such difficulties is the inability to confine the dosage form in the desired area of the gastrointestinal tract. Drug absorption from the gastrointestinal tract is a complex procedure and is subject to many variables. It is widely acknowledged that the extent of gastrointestinal tract drug absorption is related to contact time with the small intestinal mucosa (3). Thus, small intestinal transit time is an important parameter for drugs that are incompletely absorbed. Basic human physiology with the details of gastric emptying, motility patterns, and physiological and formulation variables affecting the gastric emptying are summarized.

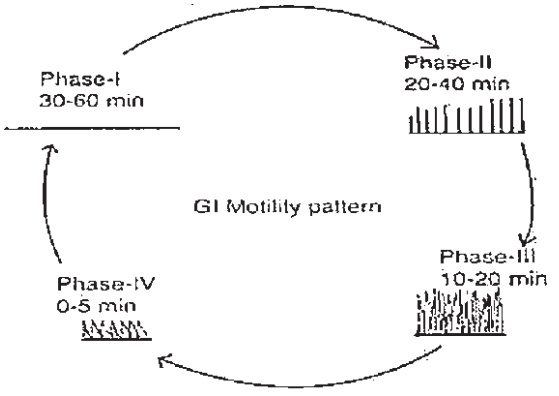
Gastroretentive systems can remain in the gastric region for several hours and hence significantly prolong the gastric residence time of drugs. Prolonged gastric retention improves bioavailability, reduces drug waste, and improves solubility for drugs that are less soluble in a high pH environment. It has applications also for local drug delivery to the stomach and proximal small intestines. Gastro retention helps to provide better availability of new products with new therapeutic possibilities and substantial benefits for patients.

The controlled gastric retention of solid dosage forms may be achieved by the mechanisms of mucoadhesion,(4,5) flotation,(6) sedimentation,(7,8) expansion,(9,10) modified shape systems,(11,12) or by the simultaneous administration of pharmacological agents(13,14) that delay gastric emptying. Based on these approaches, classification, factors affecting, approaches to design, advantages, formulation variables, and *In vivo/in vitro* evaluation of floating drug delivery systems (FDDS) has been described in detail. Several recent examples have been reported showing the efficiency of such systems for drugs with bioavailability problems.

Basic Gastrointestinal Tract Physiology

Anatomically the stomach is divided into 3 regions: fundus, body, and antrum (pylorus). The proximal part made of fundus and body acts as a reservoir for undigested material, whereas the antrum is the main site for mixing motions and act as a pump for gastric emptying by propelling actions (15).

Gastric emptying occurs during fasting as well as fed states. The pattern of motility is however distinct in the 2 states. During the fasting state an interdigestive series of electrical events take place, which cycle both through stomach and intestine every 2 to 3 hours (16). This is called the interdigestive myloelectric cycle or migrating myloelectric cycle (MMC), which is further divided into following 4 phases as described by Wilson and Washington (17). (Fig : 1)



Gastro intestinal Motility Pattern Fig.No.1

1. Phase I (basal phase) lasts from 40 to 60 minutes with rare contractions.
2. Phase II (preburst phase) lasts for 40 to 60 minutes with intermittent action potential and contractions. As the phase progresses the intensity and frequency also increases gradually.
3. Phase III (burst phase) lasts for 4 to 6 minutes. It includes intense and regular contractions for short period. It is due to this wave that all the undigested material is swept out of the stomach down to the small intestine. It is also known as the housekeeper wave.
4. Phase IV lasts for 0 to 5 minutes and occurs between phases III and I of 2 consecutive cycles.

After the ingestion of a mixed meal, the pattern of contractions change from fasted to that of fed state. This is also known as digestive motility pattern and comprises continuous contractions as in phase II of fasted state. These contractions result in reducing the size of food particles (to less than 1 mm), which are propelled toward the pylorus in a suspension form. During the fed state onset of MMC is delayed resulting in slowdown of gastric emptying rate (18).

Scintigraphic studies determining gastric emptying rates revealed that orally administered controlled release dosage forms are subjected to basically 2 complications, that of short gastric residence time and unpredictable gastric emptying rate.

Different Features of Stomach

Gastric pH : Fasted healthy subject 1.1 ± 0.15(19-21)

Fed healthy subject 3.6 ± 0.4(22)

Volume : Resting volume is about 25-50 ml

Gastric secretion: Acid, pepsin, gastrin, mucus and some enzymes about 60 ml with approximately 4 mmol of hydrogen ions per hour.

Suitable Drug Candidates For Gastroretention (23)

In general, appropriate candidates for CR-GRDf are molecules that have poor colonic absorption but are characterized by better absorption properties at the upper parts of the GIT.

- Drugs that are absorbed from the proximal part of the gastrointestinal tract (GIT).
- Drugs that are primarily absorbed from stomach and upper part of GIT
- Drugs that are less soluble or are degraded by the alkaline pH they encounter at the lower part of GIT.

TableNo.1 Salient Features Of Upper Gastrointestinal Tract:

Section	Length	Transit	pH	Microbial	Absorbing surface	Absorption
Stomach	0.2	Variable	1-4	<103	0.1	P, C, A
Small	6-10	3 ± 1	5-7.5	103 – 1010	120-200	P, C, A, F, I, E,

P – Passive diffusion C – Aqueous channel transport

A – Active transport F – Facilitated transport

I – Ion-pair transport E – Entero-or pinocytosis

CM – Carrier mediated transport

Table 2. List of Drugs Formulated as Single and Multiple Unit Forms of Floating Drug Delivery Systems

Tablets	Chlorpheniramine maleate Theophylline Furosemide Ciprofolxacin Pentoxyfillin Captopril Acetylsalicylic acid Nimodipine Amoxycillin trihydrate Verapamil HCl Isosorbide dinitrate Sotalol Atenolol Isosorbide mononitrate Acetaminophen Ampicillin Cinnarazine Diltiazem Florouracil Piretanide Prednisolone Riboflavin- 5' Phosphate

Capsules	Nicardipine L-Dopaandbenserazide Chlordiazepoxide HCl Furosemide Misoprostal Diazepam Propranlol Urodeoxycholic acid
Microspheres	Verapamil Aspirin, griseofulvin, and p-nitroaniline Ketoprofen Tranilast Iboprufen Terfenadine
Granules	Indomathacin Diclofenac sodium
Films	Cinnarizine
Powders	Several basic drugs

Table 3. Marketed Preparations of Gastro retentive technologies available in the international market(71,128)

S. No.	Product	Active Ingredient	Remarks/Type
1.	Glumetza	Metformin	Polymer Based
2.	proQuin XR	Ciprofloxacin	Polymer Based
3.	Cifran OD	Cifrofloaxacin (1 g)	Gas generating Floating Form
4.	GabapentinGR	Gabapentin (In Phase-III clinical trials)	Polymer Based
5.	-	Accordion Pill ™	Expandable film filled in capsule
6.	Baclofen GRS	Baclofen	Coated multi-layer floating & swelling system
7.	Coreg CR (Carvedilol)	Carvedilol	Gastro retention with osmotic system
8.	Madopar	Levodopa and benserzide	Floating, CR Capsule
9.	Valrelease	Diazepam	Floating Capsule
10.	Topalkan	Aluminum magnesium antacid	Floating Liquid Alginate
11.	Almagate flatcoat	Antacid	Floating Liquid Form
12.	Liquid gavison	Alginic acid and sodium bicarbonate	Effervescent floating liquid alginate preparation
13.	Cytotec	Misoprostol (100mcg/200mcg)	Bilayer Floating Capsule
14.	Conviron	Ferrous Sulphate	Colloidal gel forming FDDS

- Drugs that are absorbed due to variable gastric emptying time.
- Local or sustained drug delivery to the stomach and proximal small intestine to treat certain conditions.
- Particularly useful for the treatment of peptic ulcers caused by H. pylori infections.

Factors Affecting Gastric Retention:(24)

1. Density: GRT is a function of dosage form buoyancy that is dependent on the density. Gastric contents have a density to water (¨1.004 g/ cm ^{3}). When the patient is upright small high-density pellet sink to the bottom of the stomach (Fig: 3) they are entrapped in the folds of the antrum and withstand the peristaltic waves of the stomach wall. A density close to 2.5 g/ cm ^{3} seem necessary for significant prolongation of gastric residence time (25) and barium sulphate, zinc oxide, iron powder, titanium dioxide are used as excipients.

2. Size: Dosage form units with a diameter

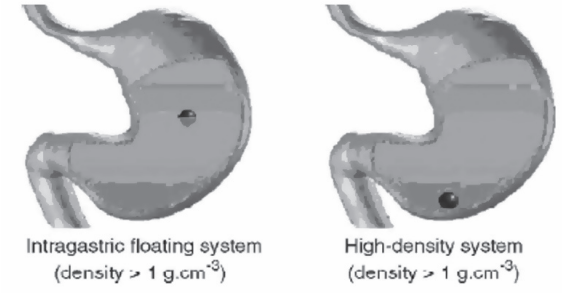


Fig No 3: Schematic Representation of an intragastric floating System and a high density system in the stomach

of more than 7.5mm are reported to have an increased GRT compared with those with a diameter of 9.9mm(26).

3. Shape of dosage form: Tetrahedron and ring shaped devices with a flexural modulus of 48 and 22.5 kilo pounds per square inch (KSI) are reported to have better GRT 90% to 100% retention at 24 hours compared with other shapes(27).

4. Single or multiple unit formulation: Multiple unit formulations show a more Predictable release profile and insignificant impairing of performance due to failure of units, allow co-administration of units with different release profiles or containing incompatible substances and permit a larger margin of safety against dosage form failure compared with single unit dosage forms.

5. Fed or unfed state: under fasting conditions: GI motility is characterized by periods of strong motor activity or the migrating myoelectric complex (MMC) that occurs every 1.5 to 2 hours. The MMC sweeps undigested material from the stomach and, if the timing of administration of the formulation coincides with that of the MMC, the GRT of the unit can be expected to be very short. However, in the fed state, MMC is delayed and GRT is considerably longer.

6. Nature of meal: feeding of indigestible polymers or fatty acid salts can change the motility pattern of the stomach to a fed state, thus decreasing the gastric emptying rate and prolonging drug release.

7. Caloric content: GRT can be increased by 4 to 10 hours with a meal that is high in proteins and fats.

8. Frequency of feed: the GRT can increase by over 400 minutes, when successive meals are given compared with a single meal due to the low frequency of MMC.

9. Gender: Mean ambulatory GRT in males (3.4±0.6 hours) is less compared with their age and race matched female counterparts (4.6±1.2 hours), regardless of the weight, height and body surface.

10. Age: Elderly people, especially those over 70, have a significantly longer GRT**.**

11. Posture: GRT can vary between supine and upright ambulatory states of the patient.

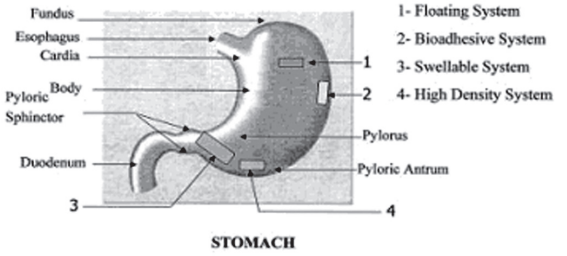
12. Concomitant drug administration: Anticholinergics like atropine and propantheline, opiates like codeine and prokinetic agents like metoclopramide and cisapride.

13. Biological factors: Diabetes and Crohn's disease.

Different Techniques Of Gastric Retention (28)

Various techniques were used to encourage gastric retention of an oral dosage form. Floating systems have low bulk density, so that they can float on the gastric juice in the stomach.2–4.The problem arises when the stomach is completely emptied of gastric fluid. In such a situation, there is nothing to float on. Different techniques used for gastric retention mentioned below: (See fig No.2)

PHYSIOLOGY OF GASTROINTESTINAL TRACT fig no. 2



STOMACH

- High density systems:
- Floating Drug delivery systems
  - ❖ Non-Effervescent systems
    - o Hydrodynamically balanced systems (HBS):
  - ❖ Effervescent systems
    - o Gas generating Systems :
    - o Low-density systems:
    - o Raft systems incorporate alginate gels:
- Expandable Systems:
- Superporous Hydrogels
- Bioadhesive or mucoadhesive systems:
- Magnetic Systems

Floating Drug Delivery Systems

The concept of FDDS was described in the literature as early as 1962. Floating drug delivery systems (FDDS) have a bulk density less than gastric fluids and so remain buoyant in the stomach without affecting the gastric emptying rate for a prolonged period of time.

While the system is floating on the gastric contents, the drug is released slowly at the desired rate from the system. After release of drug, the residual system is emptied from the stomach. This results in an increased GRT and a better control of fluctuations in plasma drug concentration (26).

Formulation of this device must comply with the following criteria:

1. It must have sufficient structure to form a cohesive gel barrier.
2. It must maintain an overall specific gravity lower than that of gastric contents (1.004 – 1.010).
3. It should dissolve slowly enough to serve as a drug reservoir.

Based on the mechanism of buoyancy, two distinctly different technologies, i.e. noneffervescent and effervescent systems, have been utilized in the development of FDDS.

Classification Of Floating Drug Delivery Systems (Fdds)

Effervescent Floating Dosage Forms

Gas Generating Systems:

These are matrix type of systems prepared with the help of swellable polymers such as methylcellulose and chitosan and various effervescent compounds, eg, sodium bicarbonate, tartaric acid, and citric acid. They are formulated in such a way that when in contact with the acidic gastric contents, CO_2 is liberated and gets entrapped in swollen hydrocolloids, which provide buoyancy to the dosage forms.

In single unit systems, such as capsules or tablets (29) effervescent substances are incorporated in the hydrophilic polymer, and CO_2 bubbles are trapped in the swollen matrix (Fig. 4a)**.** In vitro, the lag time before the unit floats is <1 min and the buoyancy is prolonged for 8 to 10 h. In vivo experiments in fasted dogs showed a mean gastric residence time increased up to 4 h. Bilayer or multilayer systems have also been designed (30). Drug and excipients can be formulated independently and the gasgenerating unit can be incorporated into any of the layers (Fig. 4b). Further refinements involve coating the matrix with a polymer which is permeable to water, but not to CO_2 (31) (Fig. 4c). The main difficulty of such formulation is to find a good compromise between elasticity, plasticity and permeability of the polymer.

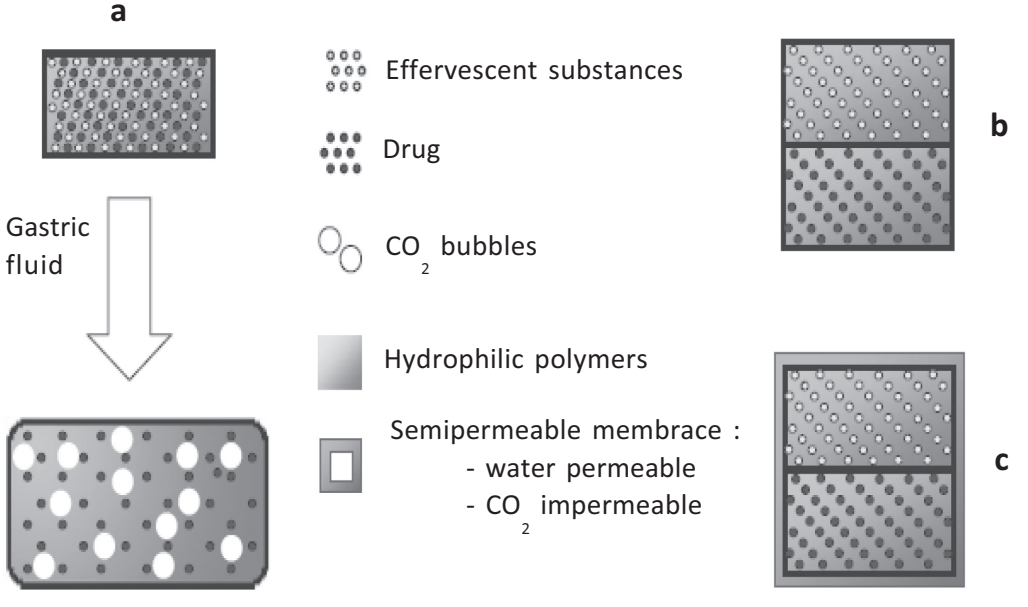


Fig No 4: Gas-generating systems. Schematic monolayer drug delivery system (a). Bilayer gas-generating systems, with (c) or without (b) semipermeable membrane.

As mentioned previously, multiple unit systems avoid the ‘‘all or nothing’’ emptying process. However, it is essential that the units remain dispersed and suspended individually in the gastric fluid and not agglomerate into a mass floating at the top of the stomach . In the beginning of the 1990s, Ichikawa et al. reported a double-layered coated system in the form of granules. It comprised an inner effervescent layer (bicarbonate and tartaric acid) and an outer swellable membrane (polyvinyl acetate and shellac). The system floated completely within 10 min and ¨80% remained floating over a period of 5 h. In vivo studies have been carried out in beagle dogs and humans in the fed state using granules loaded with barium sulphate as a radio-opaque marker. Most floated in the stomach within 10 min and remained so for at least 3 h as observed by X-ray photography (32) (Fig. 5).

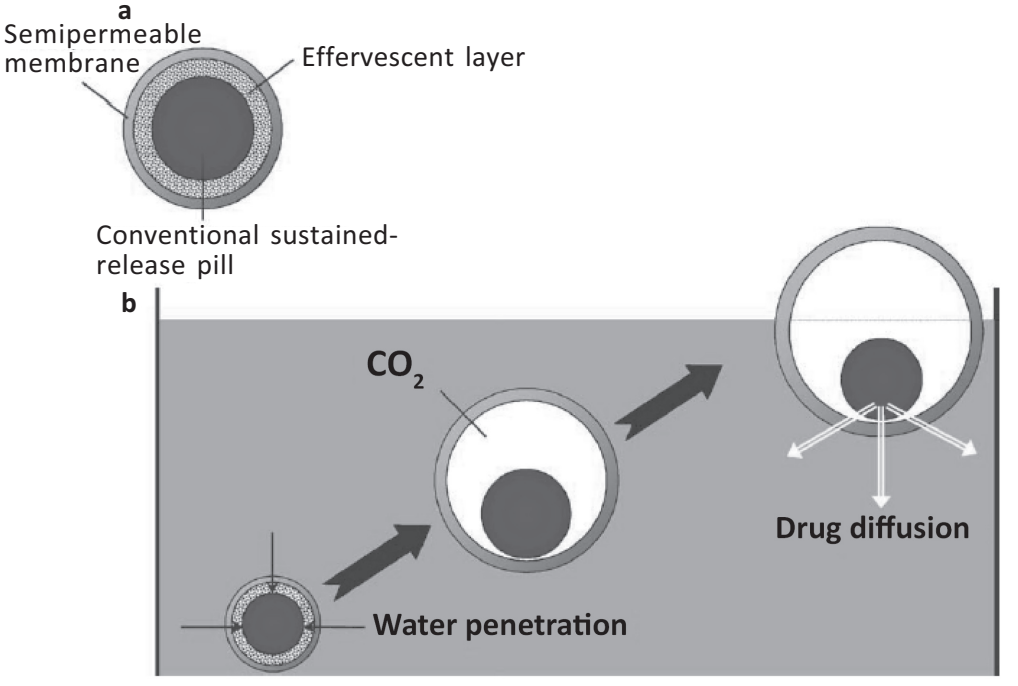


Fig No 5: Schematic representation of ‘‘floating pill’’ proposed by Ichikawa (a). The penetration of water into effervescent layer leads to a CO2 generation and makes the system float (b). Adapted from Ichikawa et al. Used with permission.

Raft-forming systems

Here, a gel-forming solution (e.g. sodium alginate solution containing carbonates or bicarbonates) swells and forms a viscous cohesive gel containing entrapped CO2 bubbles (Fig. 6) on contact with gastric fluid. Formulations also typically contain antiacids such as aluminium hydroxide or calcium carbonate to reduce gastric acidity. Because raft-forming systems produce a layer on the top of gastric fluids, they are often used for gastroesophageal reflux treatment (33-35) as with Liquid Gaviscon\ (GlaxoSmithkline).

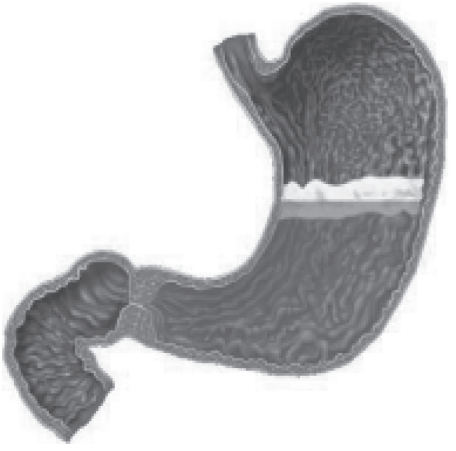


Fig No 6: Schematic illustration of the barrier formed by a raft-forming system

Low-density systems

Gas-generating systems inevitably have a lag time before floating on the stomach contents, during which the dosage form may undergo premature evacuation through the pyloric sphincter. Low-density systems (<1 g/cm3) with immediate buoyancy have therefore been developed. They are made of low-density materials, entrapping oil or air. Most are multiple unit systems, and are also called "microballoons" because of the low-density core (36) (Fig. 7a).

Streubel et al. developed foam-based floating microparticles consisting of polypropylene foam powder, drug (chlorpheniramine maleate, diltiazem HCl, theophylline or verapamil HCl) and polymer (Eudragit RS\ or polymethyl methacrylate). They were prepared by soaking the micropo-rous foam carrier with an organic solution of drug and polymer, and subsequent drying. The mixture was poured into an organic liquid (ethanol or methylene chloride) forming a suspension. The polypropylene foam particles acted like microsponges, absorbing the organic liquid, and becoming free-flowing, low-density microparticles following solvent evaporation (Fig. 7b). Good in vitro buoyancy was observed in most cases and a broad variety of drug release patterns could be achieved by varying drug loading and type of polymer: more than 77% or 98% of particles floated for at least 8 h depending on the polymer type (Eudragit RS\ or polymethyl methacrylate, respectively) and initial drug loading of the system (10% or 23%) (37).

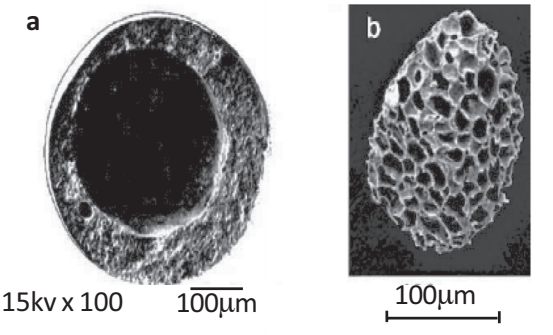


Fig No 7: Microballoons (a) from Sato et al. and foam-particles (b) from Streubel et al. Used with permissions.

Based on a similar approach, the same group developed a single unit, floating system, consisting of low-density polypropylene foam powder, matrix-forming polymers (HPMC, polyacrylates, sodium alginate, corn starch, carrageenan, agar, guar gum, and Arabic gum), drug and filler (Fig. 8). All the tablets remained floating for at least 8 h in 0.1N HCl at 37°C. The release rate could effectively be modified by varying the matrix-forming polymer/foam powder ratio, the initial drug loading, the tablet geometry (radius and height), the type of matrix-forming polymer, the use of polymer blends and

the addition of water soluble or insoluble fillers (such as lactose or microcrystalline cellulose)(38).

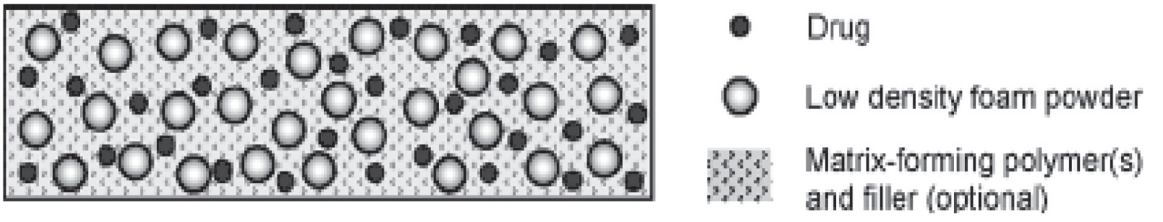


Fig No 8: Schematic presentation of the structure of the low-density, floating matrix tablets. Adapted from Streubel et al. Used with permission.

Non-Effervescent Floating Dosage Forms

Non-effervescent floating dosage forms use a gel forming or swellable cellulose type of hydrocolloids, polysaccharides, and matrix-forming polymers like polycarbonate, polyacrylate, polymethacrylate, and polystyrene. The formulation method includes a simple approach of thoroughly mixing the drug and the gel-forming hydrocolloid. After oral administration this dosage form swells in contact with gastric fluids and attains a bulk density of < 1. The air entrapped within the swollen matrix imparts buoyancy to the dosage form. The so formed swollen gel-like structure acts as a reservoir and allows sustained release of drug through the gelatinous mass.

Hydrodynamically Balanced systems

These are single-unit dosage forms, containing one or more gel-forming hydrophilic polymers. Hydroxypropylmethylcellulose (HPMC) is the most common used excipient, although hydroxyethylcellulose (HEC), hydroxypropylcellulose (HPC),sodium carboxymethylcellulose (NaCMC), agar, carrageenans or alginic acid are also used(39,40). The polymer is mixed with drug and usually administered in a gelatin capsule. The capsule rapidly dissolves in the gastric fluid, and hydration and swelling of the surface polymers produces a floating mass. Drug release is controlled by the formation of a hydrated boundary at the surface (41). Continuous erosion of the surface allows water penetration to the inner layers, maintaining surface hydration and buoyancy (39) **(Fig. 9).**

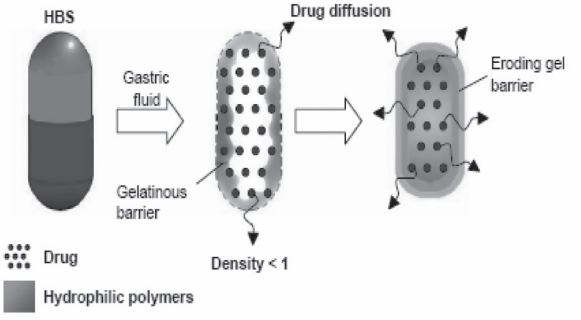


Fig No 9: Hydrodynamically balanced system (HBS). The gelatinous polymer barrier formation results from hydrophilic polymer swelling. Drug is released by diffusion and erosion of the gel barrier. Based on Hwang et al. and Dubernet . Used with permissions.

Some investigators developed bilayer formulations in which one layer conferred the buoyancy and the other controlled the drug release. Oth et al. produced a bilayer formulation

of misoprostol against gastric ulcers (42). Both layers contained swellable polymers and only one contained drug (Fig. 10a) so that buoyancy and drug release could be optimized independently. They observed a mean gastric residence time >3 h after a single meal (breakfast) and >10 h after a succession of meals.

Finally, Krogel and Bodmeier designed an impermeable polypropylene cylinder, 10–15 mm long, sealed on both sides by a matrix of hydrophilic polymer (HPMC) containing the drug. Air entrapped in the core of the cylinder provided the buoyancy (43) (Fig. 10b).

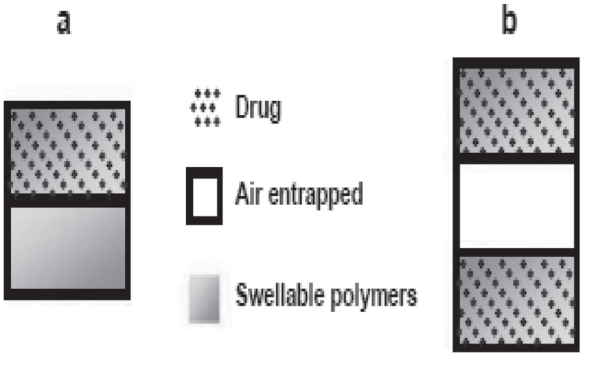


Fig No 10: Improvement in HBS proposed by Oth et al. (a) and Krogel and Bodmeier (b). Used with permissions.

Approaches To Design Floating Dosage Forms

The following approaches have been used for the design of floating dosage forms of single- and multiple-unit systems (44).

Single-Unit Dosage Forms

Various types of tablets (bilayered and matrix) have been shown to have floatable characteristics. Some of the polymers used are hydroxypropyl cellulose, hydroxypropyl methylcellulose, crosspovidone, sodium carboxymethyl cellulose, and ethyl cellulose. Self-correcting floatable asymmetric configuration drug delivery system employs a disproportionate 3-layer matrix technology to control drug release.

The use of large single-unit dosage forms sometime poses a problem of permanent retention of rigid large-sized single-unit forms especially in patients with bowel obstruction, intestinal adhesion, gastropathy, or a narrow pyloric opening (mean resting pyloric diameter 12.8 ± 7.0 mm). Floating dosage form should not be given to a patient just before going to bed as the gastric emptying of such a dosage form occurs randomly when the subject is in supine posture. One drawback of hydrodynamically balanced systems is that this system, being a matrix formulation, consists of a blend of drug and low-density polymers. The release kinetics of drug cannot be changed without changing the floating properties of the dosage form and vice versa. Single-unit formulations are associated with problems such as sticking together or being obstructed in the gastrointestinal tract, which may have a potential danger of producing irritation.

Multiple-Unit Dosage Forms

The purpose of designing multiple-unit dosage form is to develop a reliable formulation that has all the advantages of a single-unit form and also is devoid of any of the above mentioned disadvantages of single-unit formulations. In pursuit of this endeavor many multiple-unit floatable dosage forms have been designed. Microspheres have high loading capacity and many polymers have been used such as albumin, gelatin, starch, polymethacrylate, polyacrylamine, and polyalkylcyanoacrylate. Spherical polymeric microsponges also referred to as “microballoons,” have been prepared. Microspheres have a characteristic internal hollow structure and show an excellent in vitro floatability (45). In Carbon dioxide–generating multiple-unit oral formulations (46) several

devices with features that extend, unfold, or are inflated by carbon dioxide generated in the devices after administration have been described in the recent patent literature. These dosage forms are excluded from the passage of the pyloric sphincter if a diameter of ~12 to 18 mm in their expanded state is exceeded.

Effects Of Formulation Variables On The Floating Properties

Moes *et al* (39), have continuously monitored the floating kinetics to see the effect of different types of HPMC, varying HPMC/ carbopol ratio and addition of magnesium stearate on floating behavior. HBS capsules of different density were used for study. Additionof magnesium stearate was observed to improve floating property significantly. HPMC of higher grade generally exhibits a greater floating capacity; but the effect was not statistically significant for the polymers within the same viscosity K4M and E4M), the degree of substitution of the functional group did not show any significant contribution. A better floating behavior was achieved at higher HPMC: Carbopol ratio. Carbopol appeared to have negative effect on the floating behavior of FDDS.

Floating formulations using swelling polymers such as HPMC and HPC do not show reproducibility in release and residence time because the swelling depends greatly on the contents of the stomach and the osmolarity of the medium and such formulations are observed to sink in the dissolution medium after a certain time. Floating lag time with such formulation is 9-30 min. Gel-forming capacity and the gel strength of polysaccharides varies from batch to batch because of the variation in the chain length and the degree of substitution, and the situation is exacerbated in theeffervescent formulation by the disturbance of the gel structure through evolution of CO_2. In addition, gel formers react very sensitively to differences in the osmolarity of the release media, with alterations in the release (47).

Another study revealed the influence of three basic fillers [microcrystalline cellulose (MCC), dibasic calcium phosphate (DCP) and lactose] on the floating behavior of coated tablets. Tablets containing lactose floated earlier than tablets prepared with inorganic filler, DCP. Different densities could explain this; lactose-containing tablets had the lowest density 1g/cm^3 at hardness of 30 N), whereas DCP tablets had a higher density (1.9 g/ cm ^{3}at hardness of 30 N). In addition, lactose has higher water solubility and thus shows osmotic activity and faster uptake of the medium in the core of the tablet through coating. MCC, insoluble filler with a high water uptake and disintegration capability resulted in the rupturing of the coating and disintegration of the tablet, CO_2 did not accumulate under the coating and escaped through the ruptured films, floating was therefore not achieved (48).

Doelkar *et al* have showed the effect of film forming polymers on floating behavior of coated floating formulations. Films plasticized with water- soluble plasticizers are more permeable for aqueous medium but should rupture earlier than films prepared with water insoluble plasticizers. Cellulose acetate, mechanically strong polymer, is too rigid and do not expand to large extent when comes in contact with dissolution medium. Ethyl polymers mechanically weak polymer, it is not flexible and easily ruptures upon CO_2 formation; acrylic polymers are more suitable for the FDDS [43]. The floatation time decreases with increasing Eudragit RL content in Eudragit RS/RL coating and was longer with coatings containing acetyl tributyl citrate (ATBC) as plasticizer than with coating containing triethyl citrate (TEC) (49).

In Vitro And *In Vivo* Evaluation

The various parameters that need to be evaluated for their effects on GRT of buoyant formulations can mainly be categorized into following different classes:

Galenic parameters: Diametric size, flexibility and density of matrices.

Control parameters: Floating time, dissolution, specific gravity, content uniformity, hardness and friability (if tablets).

In case of multi particulate drug delivery systems, differential scanning calorimetry (DSC), particle size analysis, flow properties, surface morphology, and mechanical properties

Geometrical parameters: Shape.

Physiological parameters: Age, sex, posture and food.

The test for buoyancy and *in vitro* drug release studies are usually carried out in simulated gastric and intestinal fluids maintained at 37°C. In practice, floating time is determined by using the USP disintegration apparatus containing 900ml of 0.1N HCl as a testing medium maintained at 37°C. The time required to float the HBS dosage form is noted as floating or floatation time (1)

Dissolution tests are performed using the USP dissolution apparatus. Samples are withdrawn periodically from the dissolution medium, replenished with the same volume of fresh medium each time, and then analyzed for their drug contents after an appropriate dilution.

Burns *et al* (50) developed and validated an *in vitro* dissolution method for a floating dosage form, which had both rapid release and SR properties. The method, although based on the standard BP (1993)/ USP (1990) apparatus 2 methods, was modified such that paddle blades were positioned at the surface of dissolution medium. The results obtained with this modified paddle method showed reproducible biphasic release dissolution profiles when paddle speeds were increased from 70 to 100 rpm and the dissolution medium pH was varied (6.0-8.0). The dissolution profile was also unaltered when the bile acid concentration in the dissolution medium was increased from 7 to 14 m *M*. The specific gravity of FDDS can be determined by the displacement method using analytical grade benzene as a displacing medium (51).

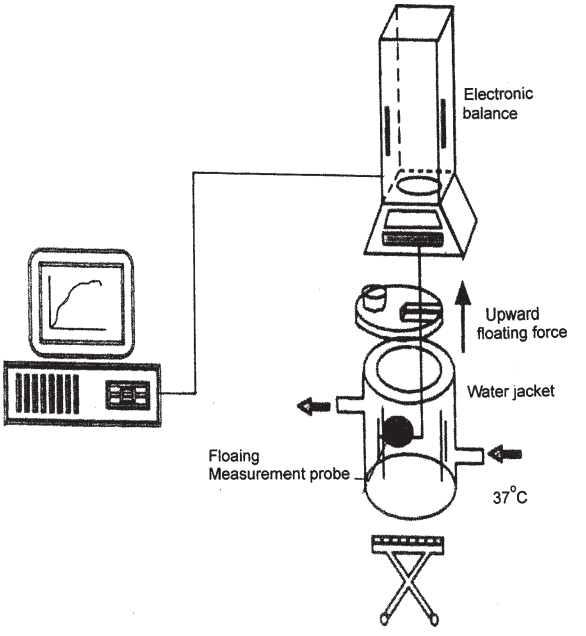


Fig No 11: Continous floating Monitoring System

The system to check continuous floating behavior (Fig 11) contains a stainless steel basket connected to a metal string and suspended from a sartorius electronic balance. The floating object is immersed at affixed depth into a water bath, which is covered to prevent water evaporation. The upward floating force could be measured by the balance and the data transmitted to an online PC through RS232 interphase using a sarto wedge program. A lotus- spread sheet could

automatically pick up the reading on the balances. Test medium used in floating kinetics measurements was 900 ml simulated gastric fluid (pH 1.2) maintained at 37°C, data was collected at 30 sec interval; baseline was recorded and subtracted from each measurement. Dissolution basket had a holder at the bottom to measure the downward force.

γ-Scintigraphy

γ-Emitting radioisotopes compounded into CR-DFs has become the state-of-art for evaluation of gastroretentive formulation in healthy volunteers. A small amount of a stable isotope e.g. Sm, is compounded into DF during its preparation. The main drawbacks of γ-scintigraphy are the associated ionizing radiation for the patient, the limited topographic information, low resolution inherent to the technique and the complicated and expensive preparation of radiopharmaceuticals (52).

Radiology

This method is the state of art in preclinical evaluation of gastroretentivity. Its major advantages as compared to γ-scintigraphy are simplicity and cost. However, use of X-ray has declined due to strict limitations, regarding the amount of exposure and it's often requirement in high quantity. A commonly used contrast agent is barium sulphate (53).

Gastroscopy

It comprises of peroral endoscopy, used with a fibereoptic and video systems. It is suggested that gastroscopy may be used to inspect visually the effect of prolonged stay in stomach milieu on the FDDS. Alternatively, FDDS may be drawn out of the stomach for more detailed evaluation (54).

Ultrasonography

Ultrasonic waves reflected substantially different acoustic impedances across interface enable the imaging of some abdominal organs (55). Most DFs do not have sharp acoustic mismatches across their interface with the physiological milieu. Therefore, Ultrasonography is not routinely used for the evaluation of FDDS. The characterization included assessment of intragastric location of the hydrogels, solvent penetration into the gel and interactions between gastric wall and FDDS during peristalsis.

Magnetic Resonance Imaging (MRI)

In the last couple of years, MRI was shown to be valuable tool in gastrointestinal research for the analysis of gastric emptying, motility and intra gastric distribution of macronutrients and drug models. The advantages of MRI include high soft tissue contrast, high temporal and spatial resolution, as well as the lack of ionizing irradiation. Also, harmless paramagnetic and supra magnetic MR imaging contrast agents can be applied to specifically enhance or suppress signal of fluids and tissues of interest and thus permit better delineation and study of organs (56).

The specific gravity of FDDS can be determined by the displacement method using analytical grade benzene as a displacing medium. The initial (dry state) bulk density of the dosage form and the changes in floating strength with time should be characterized prior to *in vivo* comparison between floating (F) and nonfloating (NF) units. Further, the optimization of floating formulations should be realized in terms of stability and durability of the floating forces produced, thereby avoiding variations in floating capability that might occur during *in vivo* studies.

Resultant weight test: An *in vitro* measuring apparatus has been conceived to determine the real floating capabilities of buoyant dosage forms as a function of time. It operates by measuring the force equivalent to the force F required to keep the object totally submerged in the fluid (57)

This force determines the resultant weight of the object when immersed and may be used to quantify its floating or nonfloating capabilities. The magnitude and direction of the force and the resultant weight corresponds to the vectorial sum of buoyancy (F_{bouy}) and gravity (F_{grav}) forces acting on the object as shown in the equation

$$F = F_{buoy} - F_{grav}$$

$$F = d_f gV - d_s gV = (d_f - d_s)\, gV$$

$$F = (df - M / V)\, gV$$

in which F is the total vertical force (resultant weight of the object), g is acceleration due to gravity, d_f is the fluid density, d_s is the object density, M is the object mass, and V is the volume of the object .By convention, a positive resultant weight signifies that the force F is exerted upward and that the object is able to float, whereas a negative resultant weight means that the force F acts downward and that the object sinks (Figure 12) (1).

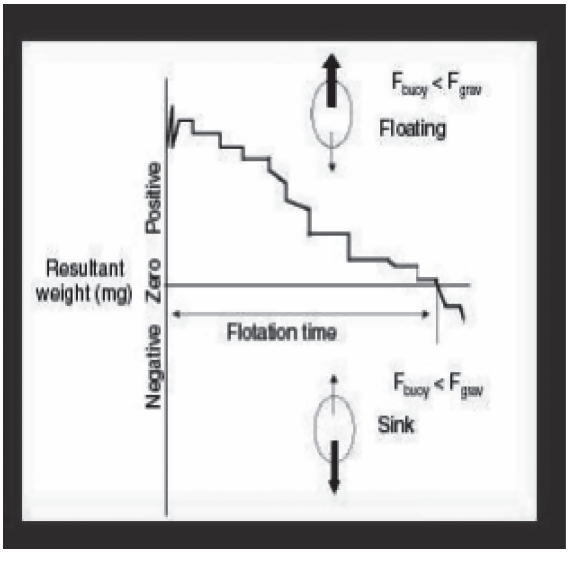


Fig No 12: Effect of resultant weight during buoyancy on the floating tendency of FDDS.

The crossing of the zero base line by the resultant weight curve from positive toward negative values indicates a transition of the dosage form from floating to nonfloating conditions. The intersection of lines on a time axis corresponds to the floating time of the dosage form.

The *in vivo* gastric retentivity of a floating dosage form is usually determined by gamma scintigraphy or roentgenography. Studies are done both under fasted and fed conditions using F and NF (control) dosage forms. It is also important that both dosage forms are non disintegrating units, and human subjects are young and healthy.

The tests for floating ability and drug release are generally performed in simulated gastric fluids at 37ºC.

In vivo gastric residence time of a floating dosage form is determined by X-ray diffraction studies, gamma scintigraphy,(58)or roentgenography (59).

Assessment of GRDF Performance *In-Vivo*

The most common laboratory animals used for absorption analysis are rats, while dogs are the more commonly used animals for evaluation of oral CR-DFs.

Prior to clinical evaluation, canine studies are usually carried out, for direct evaluation of gastroretentivity, for PK/pharmacodynamic proof-of-concept, or both. Despite some basic differences between the digestive tracts of humans and dogs, overall there are enough similarities to make the dog a useful screening tool.

The two major differences between human and dog stomach activity are the gastric emptying time after eating and the pH in the fasting state: the gastric emptying time of food in the fed state is significantly longer in dogs than in humans. In the dog, an 8-mm tablet can be

retained in the stomach for more than 8 hr following a small meal, and IMMC is abolished for about 8 hr. To prevent overestimation of the GRDF performance, the outcome of canine studies is considered as a preliminary screening prior to human studies.

Unlike the human stomach, which can reach a pH 2 at fasting state, the fasting dog's stomach pH is 5.5-6.9. Changing gastric pH of a dog may be unnecessary in evaluating nongastroretentive CR-DFs owing to their inherently short GRT.However; assessment of GRDFs may demand intervention: using gastric acidification by pentagastrin intramuscular injection, gastric-acidifying tablets, e.g., glutamic acid hydrochlor- ide, acidulin, or direct administration of acidic buffer.

Hence, dogs can be considered a good model for initial gastroretentive evaluation of a new device. On the other hand, pigs cannot be considered a proper model as they normally have a longer gastric retention of pellets and ordinary tablets than human. The most important conclusion from all previous studies is that the best model for human is human (60,61)

Pk And Pharmacodynamic Aspects Of Grdf

It has been established that the selection of a proper drug delivery system should comply with the PK and pharmacodynamic properties of the drug to ensure which input strategy would be most beneficial for achieving significant therapeutic advantages.

It seems very important to clarify a variety of PK and pharmacodynamic aspects to establish the rational selection of GRDF as the best mode of administration for certain drugs.

PK Aspects of GRDF (62)

Absorption window

An important PK aspect to be investigated when considering a GRDF candidate is the absorption of the drug along the GI tract and verifying that there is an absorption window at the upper GI tract. Various experimental techniques are available to determine intestinal absorption properties and permeability at different regions of the GI tract. In general, appropriate candidates for CR-GRDF are molecules that are characterized by better absorption properties at the upper parts of the GI tract.

Active transport mechanism

When the absorption is mediated by active transporters that are capacity limited, sustained presentation of the drug to the transporting enzymes may increase the efficacy of the transport and thus improves bioavailability.

Enhanced bioavailability

Once a narrow absorption window is defined for a compound, the possibility of improving bioavailability by continuous administration of the compound to the specific site at the upper GI tract should be tested. For example, although alendronate, levodopa, and ribo?avin are three drugs that are absorbed directly from the stomach, it was found that gastric retention of the alendronate in rats, produced by experimental/ surgical means, did not improve its bioavailability (63).

while the bioavailability of levodopa and ribo?avin was significantly enhanced when incorporated in a GRDF compared to non-GRDF CR polymeric formulations. In vivo studies remain necessary to determine the proper release profile of the drug from the DF that will provide enhanced bioavailability as different processes, related to absorption and transit of the drug in the GI tract, act concomitantly and in?uence drug absorption.

Enhanced first-pass metabolism

By increasing the GRT, a CR GRDF provides a slow and sustained input of drug to the absorption site and enhances absorption by active transporters on one hand. On the other hand, the same process may also enhance the efficiency of the presystemic metabolism by the metabolic enzymes cytochrome P450 (in particular CYP3A4 that is dominant in the gastric wall). This is an important aspect that should be taken into consideration as it means that increasing the absorption efficacy will not necessarily lead to enhanced bioavailability (63).

While CYP450 is found in the upper part of the intestine and its amount is reduced toward the colon, the P-glycoprotein (P-gp) levels increase longitudinally along the intestine and it exists in highest levels in the colon. If a drug is a P-gp substrate and does not undergo oxidative metabolism (e.g., digoxin), a CR-GRDF may improve significantly its absorption.

Elimination half-life

When drugs, with relatively short biological half-life, are introduced into a CR-GRDF, sustained and slow input is obtained, and a flip-flop PK is observed. This can enable reduced dosing frequency and consequently an improved patient compliance and therapy.

Local therapy

The continuous input of active drugs obtained from a CR-GRDF can be utilized for attaining local therapeutic concentrations for the local treatment of the stomach and the small intestine.

Pharmacodynamic Aspects of GRDF

For most of the drugs, a better delivery system would significantly minimize the? uctuations in blood levels and consequently improve the therapeutic benefit of the drug as well as reduce its concentration-dependent adverse effect. This feature is of special importance for drugs with relatively narrow therapeutic index and narrow absorption window (e.g. L-Dopa). While the continuous and sustained mode of administration attained by CR-DFs is mostly attributed to the PK advantages, there are certain pharmacodynamic aspects that have to be considered (62).

Selectivity of receptor activation

The effect of drugs that activate different types of receptors at different concentrations can be best controlled with minimization of ?uctuations and attaining ?atter drug plasma levels.

Minimized rebound activity (64)

When a given drug intervenes with natural physiological processes, it is more likely to provoke a rebound effect. This can be successfully avoided by its slow input into the blood circulation, which will minimize the counter activity and increase the drug efficacy.

Enhancing beta-lactam activity

Some drugs, such as beta-lactam antibiotics, have a nonconcentration-dependent pharmacodynamics, and their clinical response is not associated with peak concentration, but, rather, with the duration of time over a critical therapeutic concentration. SR formulations of these drugs could provide prolonged time of plasma levels over the critical concentration and enhance their efficacy.

Minimized adverse activity in the colon

As beta-lactam antibiotics have a narrow absorption window lying in the upper part of the small intestine (actively absorbed by Pept 1), a GRDF can be more beneficial as it can minimize the appearance of these compounds in the colon and thus prevent any undesirable

activities in the colon including the development of microorganism's resistance, which is an important pharmacodynamic aspect. (65, 66).

Advantages Of Gastroretentive Delivery Systems: (67)

Floating drug delivery offers several applications for drugs having poor bioavailability because of the narrow absorption window in the upper part of the gastrointestinal tract. It retains the dosage form at the site of absorption and thus enhances the bioavailability. These are summarized as follows.

- Enhanced bioavailability (68)
- Enhanced first-pass biotransformation
- Sustained Drug Delivery / reduced frequency of dosing (69)
- Site-Specific Drug Delivery (70, 71)
- Reduced fluctuations of drug concentration (72)
- Minimized adverse activity at the colon
- Absorption Enhancement (70, 73)

Works On Gastroretentive Dosage Forms

Ravichandran Mahalingam, et al (74) developed compacts containing selected bioadhesive polymers, fillers, and binders were investigated for their potential as a bioadhesive gastroretentive delivery system to deliver water soluble and water insoluble compounds in the stomach. Compacts with 90:10, 75:25, and 60:40 of polyvinylpyrrolidone (PVP) and polyethylene oxide (PEO) were evaluated for swelling, dissolution, bioadhesion, and in vitro gastric retention. Compacts containing higher PEO showed higher swelling (111.13%) and bioadhesion (0.62±0.03 N/cm2), and retained their integrity and adherence onto gastric mucosa for about 9 h under in vitro conditions. In vivo gastroretentive property of compacts were evaluated in Yorkshire cross swines. Compacts containing 58% PVP, 40% PEO and 2% of water soluble or water insoluble marker compounds showed gastroadhesive and retentive properties in vivo. It is concluded that PEO in combination with PVP yields a non disintegrating type bioadhesive dosage form which is suitable for gastroretentive applications.

Karunakar Neelam, et al (75) designed the relative bioavailability of chlorothiazide from mucoadhesive polymeric compacts is compared to commercial oral suspension in pigs. A single-dose randomized study was conducted in 12 healthy pigs that are 9–10 weeks old. After overnight fasting, pigs were divided into two groups of six animals. To the first group, a reference product containing 50 mg of chlorothiazide suspension, and in thesecond group, test product (mucoadhesive compacts) chlorothiazide (50 mg) was administered with 75 mL of water via gastric tubes. Blood samples were collected between 0 to 24 h using catheters inserted into the jugular vein. Plasma was separated by protein precipitation, and chlorothiazide concentrations were determined using a high-performance liquid chromatography method. The Tmax of mucoadhesive compacts were significantly longer ($p<0.05$; 2.17 h) than the reference products (0.58 h), whereas the Cmax of compacts were significantly lower (99 ng/mL) than the reference product (683 ng/mL; $p<0.05$). The area under the curve (AUC) of compacts accounts only 50.15% (404.32±449.93 ng h/mL) of the reference product's AUC (806.27±395.97 ng h/mL). The relative bioavailability of the compacts was lower than that of the suspension, and this may be due to the narrow window of absorption for chlorothiazide.

Lingam meka et al designed gastro retentive floating drug delivery systems for Captopril (76), Ranitidine (77) and furosemide (78) with multiple-unit mini tablets based on the gas formation

technique in order to prolong the gastric residence time and to increase the overall bioavailability of the drug. The system consists of the drug containing core units prepared by direct compression process, which are coated with three successive layers of an inner seal coat, effervescent layer (sodium bicarbonate) and an outer gas - entrapping polymeric membrane of polymethacrylates (Eudragit RL30D,RS30D and combinations of them). The formulations are evaluated for pharmacopoeial control tests and all the physical parameters evaluated were within the quality limits. The time to float decreased as amount of the effervescent agent increased and coating level of gas-entrapped polymeric membrane decreased. The drug release was controlled and linear with the square root of time. Increasing coating level of gas-entrapped polymeric membrane decreased the drug release. Both the rapid floating and the controlled release properties were achieved in the multiple-unit floating drug delivery system. The in vivo gastric residence time was examined by radiograms and it was observed that the units remained in the stomach for about 6 h.

Basak et al (79) designed floatable gastro retentive tablet of metformin hydrochloride using a gas-generating agent and gel forming hydrophilic polymer. The formulation was optimized on the basis of floating ability and *in vitro* drug release. The in vitro drug release test of these tablets indicated controlled sustained release of metformin hydrochloride and 96-99% released at the end of 8 h.

Jaimini (80) et al prepared floating tablets of famotidine employing two different grades of Methocel K100 (HPMC K100) and Methocel K15 (HPMC K15) by an effervescent echnique. These grades were evaluated for their gel-forming properties. The tablets with Methocel K100 were found to float for a longer duration compared with the formulation containing Methocel K15M. Decrease in the citric acid level increased the floating lag time. The drug release from tablets was sufficiently sustained and non-Fickian transport of the druf from tablets was confirmed.

Badve et al (81) developed hollow calcium pectinate beads for floating-pulsatile release of diclofenac sodium intended for chronopharmacotherapy. Floating pulsatile concept was applied to increase the gastric residence of the dosage form having lag phase followed by a burst release. This approach suggested the use of hollow calcium pectinate microparticles as promising floating- pulsatile drug delivery system for site- and time-specific release of drugs for chronotherapy of diseases.

Chavanpatil et al (82) developed a new gastroretentive sustained release delivery system of ofloxacin with floating, swellable and bioadhesive properties.Various release retarding polymers such as psyllium husk, HPMC K100M and a swelling agent, crosspovidone, in combinations were tried and optimized to obtain release profile over 24 h. The *in vitro* drug release followed Higuchi kinetics and the drug release mechanism was found to be non-Fickian.

Rahman et al (83) established a bilayer-floating tablet (BFT) for captopril using direct compression technology. HPMC K-grade and effervescent mixture of citric acid and sodium bicarbonate formed the floating layer. The release layer contained captopril and various polymers such as HPMC-K15M, PVP-K30 and Carbopol 934, alone or in combination with the drug. The formulation followed the Higuchi release model and showed no significant change in physical appearance, drug content, floatability or *in vitro* dissolution pattern after storage at 45 °C/75% RH for three months.

Xiaoqiang et al (84) developed a sustained release tablet for phenoporlamine hydrochloride because of its short biological half life. Three floating matrix tablets based on a gas-forming agent were prepared. HPMC K4M and Carbopol 971P were used in formulating the hydrogel system. Incorporation of sodium bicarbonate into the matrix resulted in the tablets floating over simulated gastric fluid for more than 6 hours. The dissolution profile of all the tablets showed non-Fickian diffusion in simulated gastric fluid.

Sharma et al (85) developed a multiparticulate floating pulsatile drug delivery system using porous calcium silicate and sodium alginate, for time- and site-specific drug release of meloxicam. Prepared beads were spherical with crushing strength ranging from 182 to 1073g.

Srivastava et al (86) prepared microspheres with HPMC and ethyl cellulose using solvent evaporation method.The shape and surface morphology of themicrospheres were characterized by optical and scanning electron microscopy. The microspheres exhibit prolonged drug release (8 h) and remained buoyant for more than 10 h. *In vitro* studies demonstrated diffusion-controlled drug release from the microspheres.

Chavanpatil et al (87) developed a gastroretentive dosage form for ofloxacin to be taken preferably once daily. The design of the delivery system based on a sustained release (SR) formulation with swelling and floating features in order to prolong gastric retention. Different polymers such as psyllium husk, HPMC K100M, crosspovidone and its combination were used and the formulations were evaluated for buoyancy, stability, drug content and drug release studies.

Jain et al (88) designed a controlled release system to increase GRT without contact with gastric mucosa. The was achieved through the preparation of floating microspheres by emulsion solvent diffusion technique consisting of calcium silicate (FLR) as a porous carrier, repaglinide and a Eudragit polymer. The effect of various formulation and process variables were studied.

Patel et al (89) formulated and evaluated floating tablets of ranitidine. Two fillers, namely, Avicel PH 102 and Tablettose 80 were used. It was observed that viscosity had a major influence on drug release from hydrophilic matrices as well as floating properties.

Muthusamy et al (90) designed a sustained release floating micropellets of lansoprazole by emulsion solvent diffusion technique using drug to carrier ratios of 1:1, 1:2, 1:3. HPMC, methyl cellulose and chitosan were used as carriers. The yield of micropellets was up to 82%. The drug to chitosan ratio of 1:1 showed good incorporation efficiency and high percentage *in vitro* release of lansoprazole from micropellets. The range of particle size was between 327 to 431μm.

Dave et al (91) developed gastroretentive delivery system of ranitidine HCL. Guar gum, xanthan gum and HPMC were used as gel-forming agents. Sodium carbonate was incorporated as a gas-generating agent. The effects of citric acids and stearic acid on drug release profile and floating roperties were investigated. It was indicated that a low amount of citric acid and high amount of stearic acid favoured the sustained release of ranitidine HCl.

Sato et al (92) developed microballons by emulsion solvent diffusion method using enteric acrylic polymers dissolved in a mixture of dichloromethane and ethanol. The

pharmacokinetics of riboflavin was investigated by mixing with HPMC in varying ratios, resulted in improved riboflavin release properties.

Umamaheshwari et al (93) developed floating microspheres bearing acetohydroxamic acid using polycarbonates as drug carriers by emulsion (o/w) solvent evaporation technique. The effect of polycarbonate concentration on on morphology, particle size, entrapment efficiency and drug release rate was studied.

El-Gibaly et al (94) prepared floating microcapsules containing melatonin by the interaction of chitosan and a negatively charged surfactant, sodium dioctyl sulfsuccinate. The characteristics of the floating microcapsules generated compared well with the conventional non-floating microspheres. The data obtained suggest that the floating hollow microcapsules produced would be an interesting gastroretentive controlled release delivery system for drugs.

FDDS also serves as an excellent drug delivery system for the eradication of *Helicobacter pylori*, which causes chronic gastritis and peptic ulcers. The treatment requires high drug concentrations to be maintained at the site of infection that is within the gastric mucosa. By virtue of its floating ability these dosage forms can be retained in the gastric region for a prolonged period so that the drug can be targeted (95).

Katayama et al (96) developed a sustained release (SR) liquid preparation of ampicillin containing sodium alginate, which spreads out and aids in adhering to the gastric mucosal surface. Thus, the drug is continuously released in the gastric region.

Floating microcapsules of melatonin were prepared by ionic interaction of chitosan and a surfactant, sodium dioctyl sulfosuccinate that is negatively charged. The dissolution studies of the floating microcapsules showed zero-order release kinetics in simulated gastric fluid. The release of drug from the floating microcapsules was greatly retarded with release lasting for several hours as compared with nonfloating microspheres where drug release was almost instantaneous. Most of the hollow microcapsules developed showed floating over simulated gastric fluid for more than 12 hours (97).

Sato and Kawashima (98) developed microballoons of riboflavin, which could float in JP XIII no 1 solution (simulated gastric fluid). These were prepared by an emulsion solvent technique. To assess the usefulness of the intragastric floating property of the developed microballoons of riboflavin, riboflavin powder, nonfloating microspheres of riboflavin, and floating microballoons of riboflavin were administered to 3 volunteers. Riboflavin pharmacokinetics was assessed by urinary excretion data. It could be concluded that although excretion of riboflavin following administration of floating microballoons was not sustained in fasted state, it was significantly sustained in comparison to riboflavin powder and nonfloating microspheres in the fed state. This could be due to the reason that the nonfloating formulation passes through the proximal small intestine at once from where riboflavin is mostly absorbed, while the floating microballoons gradually sank in the stomach and then arrived in the proximal small intestine in a sustained manner. Total urinary excretion (%) of riboflavin from the floating microballoons was lower than that of riboflavin powder. This was attributed to incomplete release of riboflavin from microballoons at the site of absorption.

Shimpi et al (99) studied the application of hydrophobic lipid, Gelucire 43/01 for the design of multi-unit floating systems of a highly water-soluble drug, diltiazem HCl. Diltiazem HCl-

Gelucire 43/01 granules were prepared by the melt granulation technique. The granules were evaluated for in vitro and in vivo floating ability, surface topography, and in vitro drug release. In vivo floating ability was studied by ã-scintigraphy in 6 healthy human volunteers and the results showed that the formulation remained in the stomach for 6 hours. It could be concluded that Gelucire 43/01 can be considered as an effective carrier for design of a multi-unit FDDS of highly water-soluble drugs such as diltiazem HCl.

A gastroretentive drug delivery system of ranitidine hydrochloride was designed using guar gum, xanthan gum, and hydroxy propyl methyl cellulose. Sodium bicarbonate was incorporated as a gas-generating agent. The effect of citric acid and stearic acid on drug release profile and floating properties was investigated. The addition of stearic acid reduces the drug dissolution due to its hydrophobic nature. A 3^2 full factorial design was applied to systemically optimize the drug release profile and the results showed that a low amount of citric acid and a high amount of stearic acid favor sustained release of ranitidine hydrochloride from a gastroretentive formulation. Hence, it could be concluded that a proper balance between a release rate enhancer and a release rate retardant could produce a drug dissolution profile similar to a theoretical dissolution profile of ranitidine hydrochloride (100).

In a recent work by Sriamornsak et al (101), a new emulsion-gelation method was used to prepare oil-entrapped calcium pectinate gel (CaPG) beads as a carrier for intragastric floating drug delivery. The gel beads containing edible oil were prepared by gently mixing or homogenizing an oil phase and water phase containing pectin, and then extruded into calcium chloride solution with gentle agitation at room temperature. The oil-entrapped calcium pectinate gel beads floated if a sufficient amount of oil was used. Scanning electron photomicrographs demonstrated very small pores, ranging between 5 and 40 µm, dispersed all over the beads. The type and percentage of oil played an important role in controlling the floating of oil-entrapped CaPG beads. The oil-entrapped CaPG beads were a good choice as a carrier for intragastric floating drug delivery.

Ichikawa et al (102) developed floating capsules composed of a plurality of granules that have different residence times in the stomach and consist of an inner foamable layer of gas-generating agents. This layer was further divided into 2 sublayers, the outer containing sodium bicarbonate and the inner containing tartaric acid. This layer was surrounded by an expansive polymeric film (composed of poly vinyl acetate [PVA] and shellac), which allowed gastric juice to pass through, and was found to swell by foam produced by the action between the gastric juices and the gas-generating agents. It was shown that the swellable membrane layer played an important role in maintaining the buoyancy of the pills for an extended period of time. Two parameters were evaluated: the time for the pills to be floating (TPF) and rate of pills floating at 5 hours (FP_{5h}). It was observed that both the TPF and FP_{5h} increased as the percentage of swellable membrane layer coated on pills having a effervescent layer increased. As the percentage of swellable layer was increased from 13% to 25% (wt/wt), the release rate was decreased and the lag time for dissolution also increased. The percentage of swellable layer was fixed at 13% wt/wt and the optimized system showed excellent floating ability in vitro (TPF ~10 minutes and FP_{5h} ~80%) independent of pH and viscosity of the medium.

Yang et al (103) developed a swellable asymmetric triple-layer tablet with floating ability to prolong the gastric residence time of triple drug regimen (tetracycline, metronidazole, and

clarithromycin) in *Helicobacter pylori*–associated peptic ulcers using hydroxy propyl methyl cellulose (HPMC) and poly (ethylene oxide) (PEO) as the rate-controlling polymeric membrane excipients. The design of the delivery system was based on the swellable asymmetric triple-layer tablet approach. Hydroxy-propylmethylcellulose and poly (ethylene oxide) were the major rate-controlling polymeric excipients. Tetracycline and metronidazole were incorporated into the core layer of the triple-layer matrix for controlled delivery, while bismuth salt was included in one of the outer layers for instant release. The floatation was accomplished by incorporating a gas-generating layer consisting of sodium bicarbonate: calcium carbonate (1:2 ratios) along with the polymers. The in vitro results revealed that the sustained delivery of tetracycline and metronidazole over 6 to 8 hours could be achieved while the tablet remained afloat. The floating feature aided in prolonging the gastric residence time of this system to maintain high-localized concentration of tetracycline and metronidazole (Figure 13).

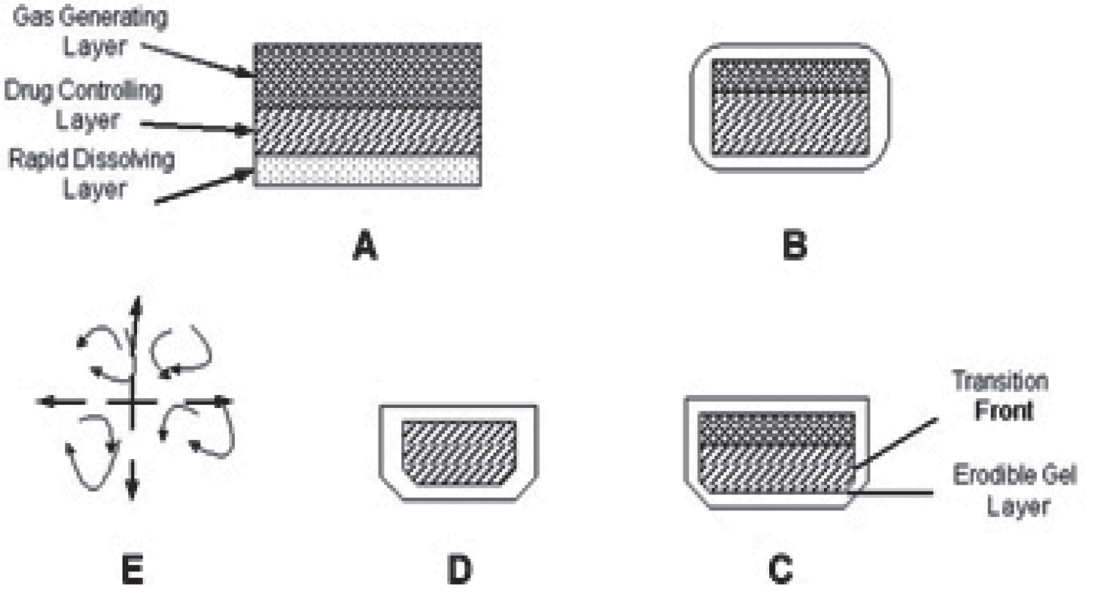


Figure 13. Schematic presentation of working of a triple-layer system. (A) Initial configuration of triple-layer tablet. (B) On contact with the dissolution medium the bismuth layer rapidly dissolves and matrix starts swelling. (C) Tablet swells and erodes. (D) and (E) Tablet erodes completely.

Ozdemir et al (104) developed floating bilayer tablets with controlled release for furosemide. The low solubility of the drug could be enhanced by using the kneading method, preparing a solid dispersion with â cyclodextrin mixed in a 1:1 ratio. One layer contained the polymers HPMC 4000, HPMC 100, and CMC (for the control of the drug delivery) and the drug. The second layer contained the effervescent mixture of sodium bicarbonate and citric acid. The in vitro floating studies revealed that the lesser the compression force the shorter is the time of

onset of floating, ie, when the tablets were compressed at 15 MPa, these could begin to float at 20 minutes whereas at a force of 32 MPa the time was prolonged to 45 minutes. Radiographic studies on 6 healthy male volunteers revealed that floating tablets were retained in stomach for 6 hours and further blood analysis studies showed that bioavailability of these tablets was 1.8 times that of the conventional tablets. On measuring the volume of urine the peak diuretic effect seen in the conventional tablets was decreased and prolonged in the case of floating dosage form.

Choi et al (105) prepared floating alginate beads using gas-forming agents (calcium carbonate and sodium bicarbonate) and studied the effect of CO_2 generation on the physical properties, morphology, and release rates. The study revealed that the kind and amount of gas-forming agent had a profound effect on the size, floating ability, pore structure, morphology, release rate, and mechanical strength of the floating beads. It was concluded that calcium carbonate formed smaller but stronger beads than sodium bicarbonate. Calcium carbonate was shown to be a less-effective gas-forming agent than sodium bicarbonate but it produced superior floating beads with enhanced control of drug release rates. In vitro floating studies revealed that the beads free of gas-forming agents sank uniformly in the media while the beads containing gas-forming agents in proportions ranging from 5:1 to 1:1 demonstrated excellent floating (100%).

Li et al (106,107) evaluated the contribution of formulation variables on the floating properties of a gastro floating drug delivery system using a continuous floating monitoring device and statistical experimental design. The formulation was conceived using taguchi design. HPMC was used as a low-density polymer and citric acid was incorporated for gas generation. Analysis of variance (ANOVA) test on the results from these experimental designs demonstrated that the hydrophobic agent magnesium stearate could significantly improve the floating capacity of the delivery system. High-viscosity polymers had good effect on floating properties. The residual floating force values of the different grades of HPMC were in the order K4 M~ E4 M~K100 LV> E5 LV but different polymers with same viscosity, ie, HPMC K4M, HPMC E4M did not show any significant effect on floating property. Better floating was achieved at a higher HPMC/carbopol ratio and this result demonstrated that carbopol has a negative effect on the floating behavior.

Fassihi and Yang (108) developed a zero-order controlled release multilayer tablet composed of at least 2 barrier layers and 1 drug layer. All the layers were made of swellable, erodible polymers and the tablet was found to swell on contact with aqueous medium. As the tablet dissolved, the barrier layers eroded away to expose more of the drug. Gas-evolving agent was added in either of the barrier layers, which caused the tablet to float and increased the retention of tablet in a patient's stomach.

Talwar et al (109) developed a once-daily formulation for oral administration of ciprofloxacin. The formulation was composed of 69.9% ciprofloxacin base, 0.34% sodium alginate, 1.03% xanthum gum, 13.7% sodium bicarbonate, and 12.1% cross-linked poly vinyl pyrrolidine. The viscolysing agent initially and the gel-forming polymer later formed a hydrated gel matrix that entrapped the gas, causing the tablet to float and be retained in the stomach or upper part of the small intestine (spatial control). The hydrated gel matrix created a tortuous diffusion path for the drug, resulting in sustained release of the drug (temporal delivery).

Two patents granted to Alza Corporation revealed a device having a hollow deformable unit that was convertible from a collapsed to

expandable form and vice versa. The deformable unit was supported by a housing that was internally divided into 2 chambers separated by a pressure-sensitive movable bladder. The first chamber contained the therapeutic agent and the second contained a volatile liquid (cyclopentane, ether) that vaporized at body temperature and imparted buoyancy to the system. The system contained a bioerodible plug to aid in exit of the unit from the body (110,111).

Baumgartner et al (112) developed a matrix-floating tablet incorporating a high dose of freely soluble drug. The formulation containing 54.7% of drug, HPMC K4 M, Avicel PH 101, and a gas-generating agent gave the best results. It took 30 seconds to become buoyant. In vivo experiments with fasted state beagle dogs revealed prolonged gastric residence time. On radiographic images made after 30 minutes of administration, the tablet was observed in animal's stomach and the next image taken at 1 hour showed that the tablet had altered its position and turned around. This was the evidence that the tablet did not adhere to the gastric mucosa. The MMC (phase during which large nondisintegrating particles or dosage forms are emptied from stomach to small intestine) of the gastric emptying cycle occurs approximately every 2 hours in humans and every 1 hour in dogs but the results showed that the mean gastric residence time of the tablets was 240 ± 60 minutes ($n = 4$) in dogs. The comparison of gastric motility and stomach emptying between humans and dogs showed no big difference and therefore it was speculated that the experimentally proven increased gastric residence time in beagle dogs could be compared with known literature for humans, where this time is less than 2 hours.

Moursy et al (113) developed sustained release floating capsules of nicardipine HCl. For floating, hydrocolloids of high viscosity grades were used and to aid in buoyancy sodium bicarbonate was added to allow evolution of CO_2. In vitro analysis of a commercially available 20-mg capsule of nicardipine HCl (MICARD) was performed for comparison. Results showed an increase in floating with increase in proportion of hydrocolloid. Inclusion of sodium bicarbonate increased buoyancy. The optimized sustained release floating capsule formulation was evaluated in vivo and compared with MICARD capsules using rabbits at a dose equivalent to a human dose of 40 mg. Drug duration after the administration of sustained release capsules significantly exceeded that of the MICARD capsules. In the latter case the drug was traced for 8 hours compared with 16 hours in former case.

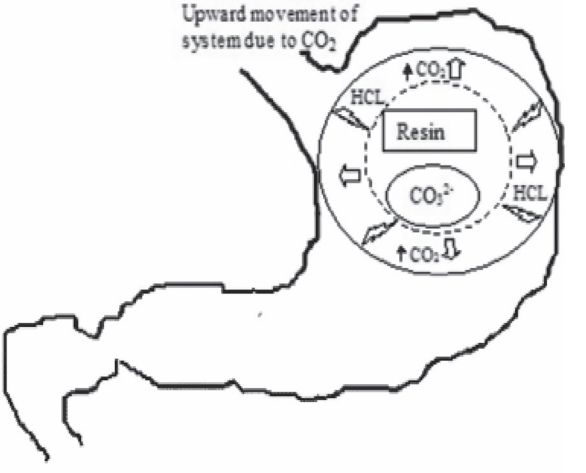


Figure 14. Pictorial presentation of working of effervescent floating drug delivery system based on ion exchange resin.

Atyabi and coworkers et al (114) developed a floating system using ion exchange resin that was loaded with bicarbonate by mixing the beads with 1 M sodium bicarbonate solution. The loaded beads were then surrounded by a semipermeable membrane to avoid sudden loss of CO_2. Upon coming in contact with gastric contents an

exchange of chloride and bicarbonate ions took place that resulted in CO_2 generation thereby carrying beads toward the top of gastric contents and producing a floating layer of resin beads (Figure 14) .The in vivo behavior of the coated and uncoated beads was monitored using a single channel analyzing study in 12 healthy human volunteers by gamma radio scintigraphy. Studies showed that the gastric residence time was prolonged considerably (24 hours) compared with uncoated beads (1 to 3 hours).

Thanoo et al (115) developed polycarbonate microspheres by solvent evaporation technique. Polycarbonate in dichloromethane was found to give hollow microspheres that floated on water and simulated biofluids as evidenced by scanning electron microscopy (SEM). High drug loading was achieved and drug-loaded microspheres were able to float on gastric and intestinal fluids. It was found that increasing the drug-to-polymer ratio increased both their mean particle size and release rate of drug.

Nur and Zhang et al (116) developed floating tablets of captopril using HPMC (4000 and 15 000 cps) and carbopol 934P. In vitro buoyancy studies revealed that tablets of 2 kg/cm^2 hardness after immersion into the floating media floated immediately and tablets with hardness 4 kg/cm^2 sank for 3 to 4 minutes and then came to the surface. Tablets in both cases remained floating for 24 hours. The tablet with 8 kg/cm^2 hardness showed no floating capability. It was concluded that the buoyancy of the tablet is governed by both the swelling of the hydrocolloid particles on the tablet surface when it contacts the gastric fluids and the presence of internal voids in the center of the tablet (porosity). A prolonged release from these floating tablets was observed as compared with the conventional tablets and a 24-hour controlled release from the dosage form of captopril was achieved.

Bulgarelli et al (117) studied the effect of matrix composition and process conditions on casein gelatin beads prepared by emulsification extraction method. Casein by virtue of its emulsifying properties causes incorporation of air bubbles and formation of large holes in the beads that act as air reservoirs in floating systems and serve as a simple and inexpensive material used in controlled oral drug delivery systems. It was observed that the percentage of casein in matrix increases the drug loading of both low and high porous matrices, although the loading efficiency of high porous matrices is lower than that of low porous matrices.

Fell et al (118) prepared floating alginate beads incorporating amoxycillin. The beads were produced by dropwise addition of alginate into calcium chloride solution, followed by removal of gel beads and freeze-drying. The beads containing the dissolved drug remained buoyant for 20 hours and high drug-loading levels were achieved.

Asmussen et al (119) invented a device for the controlled release of active compounds in the gastrointestinal tract with delayed pyloric passage, which expanded in contact with gastric fluids and the active agent was released from a multiparticulate preparation. It was claimed that the release of the active compound was better controlled when compared with conventional dosage forms with delayed pyloric passage.

El-Kamel et al (120) prepared floating microparticles of ketoprofen, by emulsion solvent diffusion technique. Four different ratios of Eudragit S 100 with Eudragit RL were used. The formulation containing 1:1 ratio of the 2 above-mentioned polymers exhibited high percentage of floating particles in all the examined media as evidenced by the percentage of particles floated at different time intervals. This can be attributed to the low bulk density, high packing velocity, and high packing factor.

Illum and Ping et al (121) developed microspheres that released the active agent in the stomach environment over a prolonged period of time. The active agent was encased in the inner core of microspheres along with the rate-controlling membrane of a water-insoluble polymer. The outer layer was composed of bioadhesive (chitosan). The microspheres were prepared by spray drying an oil/water or water/oil emulsion of the active agent, the water-insoluble polymer, and the cationic polymer.

Sheth and Tossounian (122) developed hydrodynamically balanced sustained release tablets containing drug and hydrophilic hydrocolloids, which on contact with gastric fluids at body temperature formed a soft gelatinous mass on the surface of the tablet and provided a water-impermeable colloid gel barrier on the surface of the tablets. The drug slowly released from the surface of the gelatinous mass that remained buoyant on gastric fluids (Figure 15, A and B).

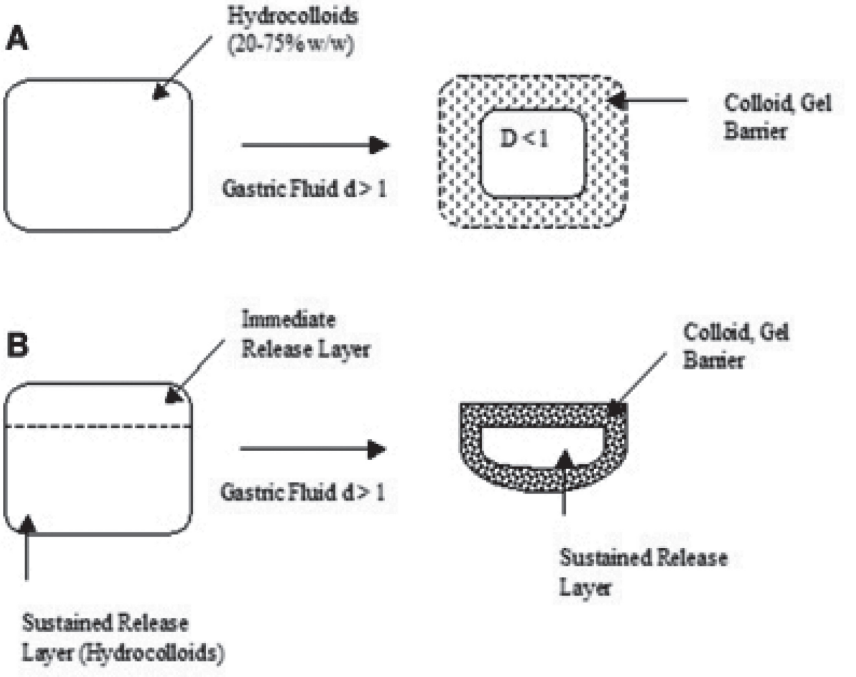


Figure 15. Intragastric floating tablets. (A) United States patent 4 167 558, September 11, 1979. (B) United States patent 4 140 755, February 20, 1979.

Spickett et al (123) invented an antacid preparation having a prolonged gastric residence time. It comprised 2 phases. The internal phase consisted of a solid antacid and the external phase consisted of hydrophobic organic compounds (mono-, di-, and triglycerides) for floating and a non-ionic emulsifier.

Franz and Oth (124) described a sustained release dosage form adapted to release of the drug over an extended period of time. It comprised a bilayer formulation in which one layer consisted of drug misoprostal and the other had a floating layer. The uncompressed bilayer formulation was kept in a capsule and was shown

to be buoyant in the stomach for 13 hours. The dosage form was designed in such a way that all the drug was released in the stomach itself.

Wu et al (125) developed floating sustained release tablets of nimodipine by using HPMC and PEG 6000. Prior to formulation of floating tablets, nimodipine was incorporated into poloxamer-188 solid dispersion after which it was directly compressed into floating tablets. It was observed that by increasing the HPMC and decreasing the PEG 6000 content a decline in in vitro release of nimodipine occurred.

Wong et al (126) developed a prolonged release dosage form adapted for gastric retention using swellable polymers. It consisted of a band of insoluble material that prevented the covered portion of the polymer matrix from swelling and provided a segment of a dosage form that was of sufficient rigidity to withstand the contractions of the stomach and delayed the expulsion of the dosage form from the stomach.

Yang et al (127) developed a swellable asymmetric triple-layer tablet with floating ability to prolong the gastric residence time of triple drug regimen (tetracycline, metronidazole, clarithromycin) of *Helicobacter pylori*–associated peptic ulcers using HPMC and PEO as the rate-controlling polymeric membrane excipients. Results demonstrated that sustained delivery of tetracycline and metronidazole over 6 to 8 hours could be achieved while the tablets remained floating. It was concluded that the developed delivery system had the potential to increase the efficacy of the therapy and improve patient compliance.

Limitations

Floating drug delivery is associated with certain limitations.More predictable and reproducible floating properties should be achieved in all the extreme gastric conditions.

- They require a sufficiently high level of fluids in the stomach for the drug delivery buoyancy, to float therein and to work efficiently.
- Drugs that irritate the mucosa, those that have multiple absorption sites in the gastrointestinal tract, and those that are not stable at gastric pH are not suitable candidates to be formulated as floating dosage forms.
- Floating systems are not feasible for those drugs that have solubility or stability problems in gastric fluid.
- Drugs such as Nifedipine, which is well absorbed along the entire GI tract and which undergoes significant first- pass metabolism, may not be desirable candidates for FDDS since the slow gastric emptying may lead to reduced systemic bioavailability.
- Also there are limitations to the applicability of FDDS for drugs that are irritant to gastric mucosa.
- The floating systems in patients with achlorhydria can be questionable in case of swellable systems, faster swelling properties are required and complete swelling of the system should be achieved well before the gastric emptying time.
- Bioadhesion in the acidic environment and high turnover of mucus may raise questions about the effectiveness of this technique. Similarly retention of high density systems in the antrum part under the migrating waves of the stomach is questionable.
- Not suitable for drugs that may cause gastric lesions e.g. Non- steroidal anti

inflammatory drugs. Drugs that are unstable in the strong acidic environment, these systems do not offer significant advantages over the conventional dosage forms for drugs, which are absorbed throughout the gastrointestinal tract.

- The mucus on the walls of the stomach is in a state of constant renewal, resulting in unpredictable adherence.
- In all the above systems the physical integrity of the system is very important and primary requirement for the success of these systems.
- Floatation as a retention mechanism requires the presence of liquid on which the dosage form can float on the gastric contents. To overcome this limitation, a bioadhesive polymer can be used to coat the dosage so that it adheres to gastric mucosa [83], or the dosage form can be administered with a full glass of water to provide the initial fluid for buoyancy. Also single unit floating capsules or tablets are associated with an "all or none concept," but this can be overcome by formulating multiple unit systems like floating microspheres or microballoons [84].

Conclusion

Drug absorption in the gastrointestinal tract is a highly variable procedure and prolonging gastric retention of the dosage form extends the time for drug absorption. FDDS promises to be a potential approach for gastric retention. Dosage forms with a prolonged GRT will bring about new and important therapeutic options. They will significantly extend the period of time over which drugs may be released and thus prolong dosing intervals and increase patient compliance beyond the compliance level of existing CRDFs. Many of the "Once-a-day" formulations will be replaced by products with release and absorption phases of approximately 24 hrs. Also, GRDFs will greatly improve the pharmacotherapy of the stomach itself through local drug release leading to high drug concentrations at gastric mucosa which are sustained over a large period. Finally, GRDFs will be used as carriers of drugs with the "absorption window". Replacing parenteral administration of drugs to oral pharmacotherapy would substantially improve treatment. It is anticipated that FDDS may enhance this possibility. Moreover, it is expected that the FDDS approach may be used for many potential active agents with a narrow absorption window, whose development has been halted due to the lack of appropriate pharmaceutical FDDS technologies. Combination therapy to treat *H. pylori* infection in a single FDDS needs to be developed. Although there are number of difficulties to be worked out to achieve prolonged gastric retention, a large number of companies are focusing toward commercializing this technique.

Future Prospects

While the control of drug release profiles has been a major aim of pharmaceutical research and development in the past three decades, the control of GI transit profiles could be the focus of the next two decades and might result in the availability of new products with new therapeutic possibilities and substantial benefits for patients. Soon, the so-called 'once-a-day' formulations may be replaced by novel gastroretentive products with release and absorption phases of approximately 24 hours.

Further investigations may concentrate on the following concepts:

Identification of a minimal cut-off size above that DFs retained in the human stomach for prolonged periods of time. This would permit a more specific control to be achieved in gastroretentivity.

Design of an array of FDDS, each having a narrow GRT for use according to the clinical need e.g. dosage and state of disease. This may be achieved by compounding polymeric matrices with various biodegradation properties.

Study of the effect of various geometric shapes, in a more excessive manner than previous studies, extended dimensions with high rigidity, on gastroretentivity.

Design of novel polymers according to clinical and pharmaceutical need.

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Protective effects of Calcium and Zinc on Lead induced perturbations in brain acetylcholinesterase activity

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Abstract

Alteration in the cholinergic system is one of the major pathological hallmarks of lead (Pb) toxicity. Pb-exposure alters acetylcholinesterase (AChE) activity in brain regions which control learning and cognitive behavior. The present study is focused on the beneficial effects of calcium (Ca^{2+}) and zinc (Zn^{2+}) supplementation on Pb-induced alterations in AChE activity in the brains of developing and adult mice. Mice were lactationally exposed to Pb (0.2% and 1%) and supplemented with either calcium (Ca^{2+}) or zinc (Zn^{2+}) and the synaptosomal AChE enzyme activity was analyzed in cortex, hippocampus, cerebellum and medulla of brains excised on postnatal day (PND) 14, 21, 28 and 3 months. The histochemical staining for AChE was also performed in the sections of mice brain. Exposure to Pb resulted in a significant decrease in the activity and staining intensity of AChE. Interestingly the supplementation with Ca^{2+} or Zn^{2+} reversed the Pb-induced effects on AChE activity and expression. These findings strongly support that calcium and zinc supplementation significantly protects the brain from the Pb-induced alterations in cholinergic system.

Keywords: lead, calcium, zinc, acetylcholinesterase, synaptosomes

Introduction

Inorganic lead (Pb) represents a long lived and persistent environmental hazard to the nervous system. It has been proposed that cholinergic neurotransmitter system may be involved in the pathophysiology of Pb poisoning (34, 35, 52). One principal constellation of Pb toxicity includes alterations in motor coordination and cortical function and as such, draws attention to Pb mediated impairments in cholinergic neurotransmission (30). Conflicting effects of Pb on acetylcholinesterase (AChE) have been reported by several authors. Tomlinson et al., (56) have shown the inhibitory effects of Pb on AChE activity in vitro, however, Modak et al., (35) found that AChE activity was unchanged in the cortex and cerebellum of rats exposed to Pb before and after weaning. A generalized reduction in brain cholinergic function has been reported in Pb-treated mice (8) and rats (1). The sensitivity of the brain regions to Pb was further supported by the in vitro studies conducted using different concentrations of Pb, which showed dose dependent inhibitory actions on AChE activity in a manner similar to the presence of eserine (47).

Several authors linked the reduction of AChE in the hippocampus region with short-term memory disturbance, which could result in incomplete understanding and consequently, abnormal communication skills and stated that exposure to Pb decreases the levels of acetylcholine causing less than one-fifth of normal concentration and a strong negative IQ (51). Schwartz et al., (50) suggested that cognitive function can progres-

sively decline as a result of past occupational exposures to a neurotoxicant. Exposure to Pb even at low concentrations; impairs both sensory and motor functions (32).

Since Pb is an element, its neurotoxic actions might be due to interactions with other essential elements. It has been suggested that Pb might compete with other elements, especially divalent and monovalent cations such as calcium (Ca^{2+}), magnesium (Mg^{2+}), sodium (Na^{+}) and iron (Fe^{2+}). Ca^{2+}-Pb interactions are also related to important clinical effects of Pb both in the cell and at molecular level. Pb blocks entry of Ca^{2+} into nerve terminals. Pb negatively affects normal Ca^{2+} balance in cells, which is essential for normal cell function. Zinc (Zn^{2+}) counteracts the inhibitory action of Pb on hematopoietic enzymes. Interactions between Zn^{2+} and Pb have been investigated at absorptive and enzymatic sites (18). Zn^{2+} and Pb compete for similar binding sites on the metallothionein like transport protein in the gastrointestinal tract (25). The competition between Zn^{2+} and Pb might decrease the absorption of Pb, thus reducing Pb toxicity.

In the present study, we investigated the extent of dose related Pb effects on AChE activity in different regions of mouse brain and the extent to which Ca^{2+} / Zn^{2+} supplementation has protection over Pb-induced cholinergic perturbations.

Materials and Methods

***Materials*:** All chemicals used in the study were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Animals: Pregnant Swiss albino mice were obtained from Indian Institute of Science, Bangalore. The animals were housed in clear plastic cages with hardwood bedding in the animal facility and maintained at 28 ± 2 °C and relative humidity 60 ± 10% with a 12 h light/day cycle. Standard mice chow (Sai Durga feeds and foods, Bangalore, India) and water were made available ad libitum. The protocol and animal use were approved by Institution Animal Ethical Committee, S.V. University.

***Animal exposure*:** Mice were lactationally exposed to 0.2% and 1% Pb by adding Pb-acetate to deionized drinking water of the mother. Pb-exposure dose was obtained from the published work and our earlier studies (13, 42 - 45, 48, 60). Pb-exposure commenced on PND 1, continued up to PND 21 and stopped at weaning. Litters consisting of eight males were randomly selected and placed with each dam. Control mice received only deionized water.

Calcium and zinc supplementation: Calcium (Ca^{2+}) or Zinc (Zn^{2+}) was supplemented as 0.02% in 0.2% Pb-water and 0.1% in 1% Pb-water and is separately given to the mothers up to PND 21 and stopped at weaning. The supplemented doses and protocols were obtained from previously published works (5, 42 - 45).

Isolation of tissues: Control, Pb-treated and Ca^{2+}/Zn^{2+} supplemented mice were sacrificed by cervical decapitation at PND 14, PND 21, PND 28 and 3months. After decapitation, the brain was removed onto ice-cold glass plate and different brain regions (cortex, hippocampus, cerebellum and medulla) were quickly dissected, snap frozen on dry-ice and stored at -80 °C until use. The postnatal time periods were selected from published works and based on developmental differentiation and cellular maturation (5, 42 – 45, 54).

Isolation of synaptosomal fractions: Synaptosomal fractions were isolated from brain homogenates of cortex, hippocampus, cerebellum and medulla using Ficoll - sucrose gradients (12). The cerebral cortex, cerebellum, hippocampus and medulla were isolated in cold conditions. The

tissues were weighed and homogenized in 10 ml of ice-cold homogenizing buffer and the volume was brought up to 25 mL with homogenizing buffer. The homogenates were centrifuged at 750g for 10 minutes. The pellets were discarded. The supernatants were centrifuged at 17,000g for 20 minutes. The pellets were suspended in 10 ml of 0.32 M sucrose and were layered on a two step discontinuous Ficoll-sucrose gradient consisting 13% and 7.5% Ficoll solution and centrifuged at 65,000g for 45 minutes. The milky layer was formed at the interface of 13% and 7.5% Ficoll. The milky layer fraction was diluted with 9 volumes of 0.32 M sucrose and centrifuged again 17,000g for 30 minutes. The supernatant was discarded and the pellet (synaptosomal fraction) was suspended in 0.32 M sucrose and used for enzyme assay.

Estimation of acetylcholinesterase activity (AChE) (EC 3.1.1.7): AChE activity was estimated following the method of Ellman et al., (15). 2% (w/v) tissue homogenates were prepared in 0.25 M sucrose solution. The reaction mixture contained 3.0 ml of phosphate buffer (pH 8.0), 20 µl of 0.075 M acetylcholine iodide (substrate) and 100 µl of 0.01 M 5, 5-Dithiobis-2-Nitrobenzoic acid (DTNB). The reaction was initiated with the addition of 100 µl of crude homogenate/synaptosomal fraction. The contents were incubated for 30 minutes at room temperature and the color absorbance was measured at 412 nm in a UV/VIS spectrophotometer (Hitachi, Model U-2000) against a blank. The enzyme activity was expressed as µ moles of ACh hydrolyzed/mg protein/hr.

AChE Histochemistry: AChE histochemistry was performed as described by Hedreen et al., (22), modified method of Karnovsky and Roots (26). Mice of one month old were anasthetized with sodium pentoborbitol and perfused with 50 mM phosphate buffered saline (PBS, pH 7.4, 4º C) followed by 4% paraformaldehyde (4º C) through cardiac catheter. Brains were removed and postfixed in paraformaldehyde for 2 hours. This was followed by cryoprotection in 10, 20, and 30% sucrose gradients. Eight micron thick sections were cut in a freezing monotome (Cryostat, Bright Instrument Company Ltd, England). The sections were rinsed in 0.1 M phosphate buffer (pH 6.0) and incubated in a solution containing 32.5 ml of 0.1 M phosphate buffer (pH 6.0); 2.0 ml of 0.1 M sodium citrate; 5 ml of 0.03 M cupric sulphate; 1.0 ml of 0.0005 M potassium ferricyanide; 25 mg of acetylthiocholine iodide and 9.5 ml of distilled water. Sections were also incubated with the AChE inhibitor, eserine (10^{-4} M), in the above media to study the inhibitory effect and non-specific staining. The sections were incubated for 20 minutes at room temperature and then dehydrated in ethanol series, cleared in xylene and mounted in permount.

Estimation of protein content: Protein content of the tissues was estimated by the method of Lowry et al., (29).

Statistical analysis of the data: The data obtained from six separate samples were expressed as mean ± SD. Significance between various age groups and treatments was analyzed by two-way analysis of variance (ANOVA). Significance of each age group among different treatments was analyzed by one-way analysis of variance (ANOVA) followed by Newman–Keuls Multiple comparison post hoc test using Standard Statistical Software Package to compare the effects among various groups. The 0.05 level of probability was used as the criterion for significance.

Results

The specific activity of AChE was determined in the synaptosomal fractions of cerebral cortex, hippocampus, cerebellum and medulla

regions of control, Pb-exposed (low and high doses) and Ca^{2+}/ Zn^{2+}supplemented mice at different postnatal periods (PND 14, 21, 28) and at 3 month age.

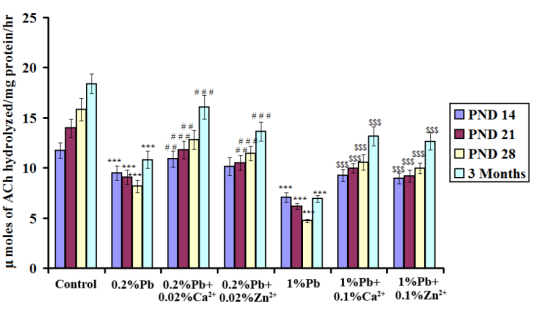
The results of the present study showed that the specific activity of AChE was significantly higher in the synaptosomal fraction of hippocampus followed by cerebral cortex, cerebellum and medulla. The activity levels of AChE were significantly increased from PND 14 to 3 months in an age dependent manner. In the synaptosomal fractions of various regions of control brain, the specific activity was found to be 11.74, 13.96, 15.82 and 18.38 µ moles of ACh hydrolyzed/mg protein/hr in cortex (Fig 1a); 13.2, 15.12, 17.16 and 19.47 in hippocampus(Fig 1b); 10.46, 12.28, 14.82 and 17.52 in cerebellum region (Fig 1c) and 9.72, 11.61, 13.62 and 15.2 in medulla region (Fig 1d) at PND 14, PND 21, PND 28 and 3 month respectively.

The 0.2% Pb-exposure resulted in a decrease of 59.59% in hippocampus, followed by cerebellum (56.27%), medulla (53.93%) and cerebral cortex (48.50%) in the synaptosomal AChE. The inhibitory effect of high dose (1%) Pb was more in cerebral cortex (70.20%) followed by hippocampus (68.70%), cerebellum (66.40%) and medulla (64.70%) (Fig 1).

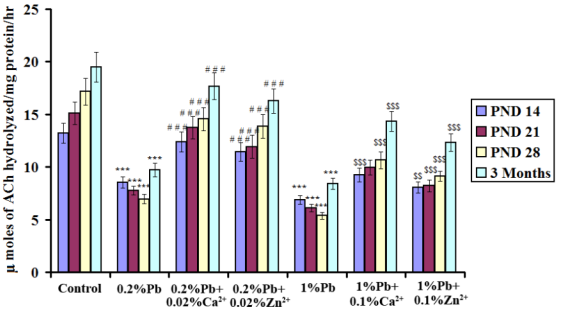
The supplementation with Ca^{2+}/Zn^{2+} reversed the inhibitory effect caused by Pb-exposure on AChE activity in synaptosomal fractions. A maximum recovery of 57% in cortex, 110% in hippocampus, 72% in cerebellum and 73% in medulla from 0.2% Pb-induced inhibition on AChE activity was observed when 0.02% Ca^{2+} was supplemented with 0.2% Pb where as a recovery of 40% in cortex, 100% in hippocampus, 68% in cerebellum and 51% in medulla was observed with 0.02% Zn^{2+} supplementation to 0.2% Pb. A recovery of 124%, 98%, 87%, 106% in cortex, hippocampus, cerebellum and medulla respectively was observed with 0.1% Ca^{2+} supplementation to 1% Pb and a recovery of 111%, 70%, 72% and 54% was observed in cortex, hippocampus, cerebellum and medulla respectively from 1% Pb-induced inhibition on AChE activity when 0.1% Zn^{2+} was supplemented (Fig 1).

Fig.1

a)



b)



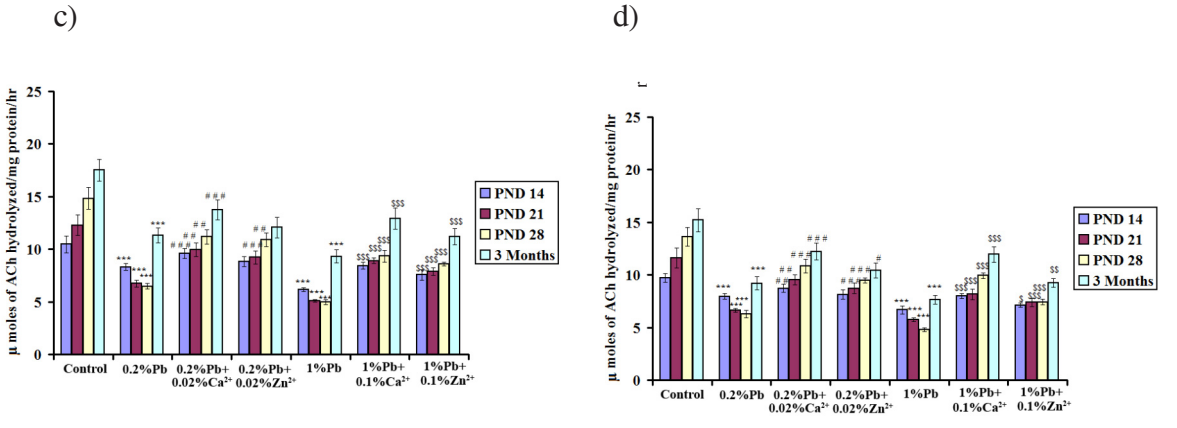


Fig. 1. Effect of Pb-exposure and calcium/zinc supplementation to Pb on acetylcholinesterase (AChE) activity in cerebral cortex (a), hippocampus (b), cerebellum (c) and medulla (d) regions of mouse brain. Male mice were exposed to either deionized water (control) or Pb-acetate (0.2% or 1%) or calcium and zinc together with Pb (0.02% in 0.2% Pb and 0.1% in 1% Pb in deionized water) from PND 1 through PND 21. AChE activity was determined in brain regions in PND 14, PND 21, PND 28 and 3 months old control, Pb-exposed, calcium/zinc supplemented mice. The enzyme activity was expressed as µ moles of ACh hydrolyzed/mg protein/hr. Values are mean ± SD of six separate experiments. Significance of each age group among different treatments was analyzed by one-way analysis of variance (ANOVA) followed by Newman–Keuls Multiple comparison post hoc test. The 0.05 level of probability was used as the criterion for significance. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to controls, #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ compared to 0.2% Pb treatment and \$$p < 0.05$, \$\$$p < 0.01$, \$\$\$$p < 0.001$ compared to 1% Pb treatment.

Both Ca^{2+}/Zn^{2+} supplementation was found to be protective in reducing the Pb-burden. The supplementation of Ca^{2+} or Zn^{2+} to low dose Pb-exposure appears to be more effective than to high dose Pb. As compared to Zn^{2+}, the Ca^{2+} supplementation was found to be more protective (Fig 1).

Histochemical staining of AChE revealed regional alterations in the brain of Pb-exposed mouse in a dose dependent manner. However, the alterations in AChE staining with 0.2% Pb-exposure were not significantly visible as compared to high level Pb-exposure. The AChE histochemical staining appeared intensely in cortical cell layers (Fig 2 (I)), dentate gyrus and CA3 of hippocampus (Fig 2 (II)), and molecular and granule cell layers of cerebellum (Fig 2 (III)) of control mouse (A). Pb-exposure (B) decreased the staining markedly in CA3 / CA2 areas and dentate gyrus of hippocampus (Fig 2 (II)). Pb also produced decrease in staining in molecular and granular cell layers of cerebellum and cortical layers (Fig 2). The supplementation with Ca^{2+}/Zn^{2+} (C and D) increased AChE decreasing the Pb-burden on AChE staining (Fig 2).

Fig.2

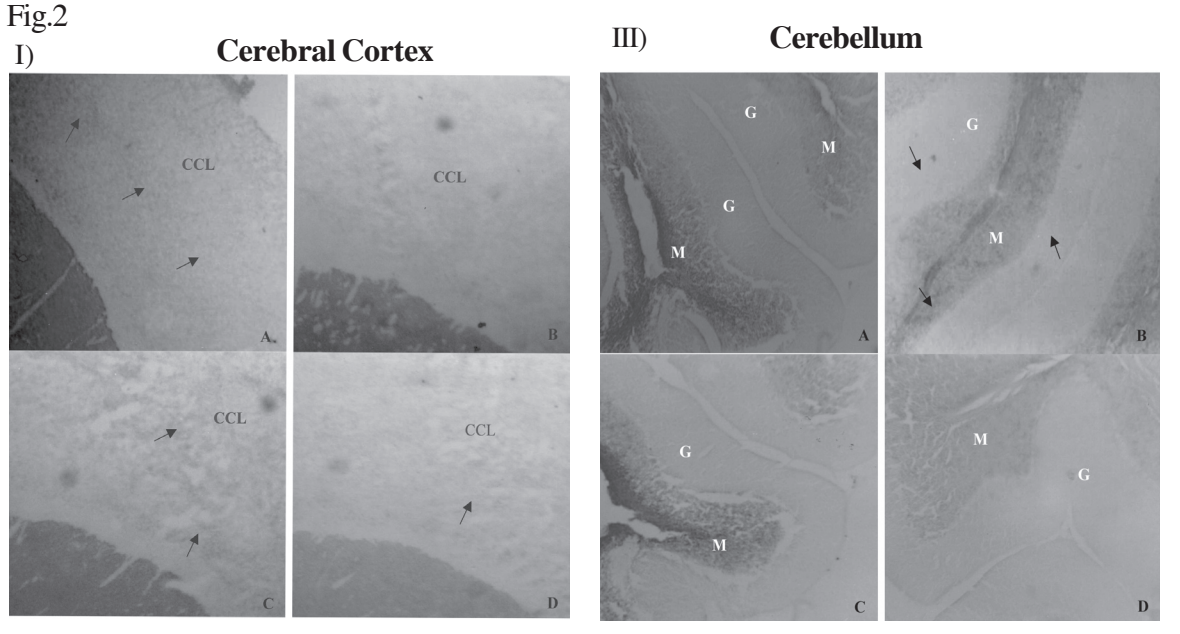


Fig. 2. Alterations in histochemical staining for AChE in Pb-exposed and calcium/zinc supplemented cerebral cortex (I), hippocampus (II) and cerebellum (III) of control (A), 1% Pb-exposed (B), 1% Pb+ 0.1% Ca^{2+} (C) and 1% Pb+ 0.1% Zn^{2+}(D) of one month old mouse brain. Male mice were exposed to either deionized water (control) or Pb-acetate (1%) or calcium and zinc together with Pb (0.1% in 1% Pb in deionized water) from PND 1 through PND 21. AChE staining was performed in control, Pb-exposed, calcium/zinc supplemented mice brain sections of eight micron thickness cut in a cryostat. Areas marked with (→) show the changes in the intensity of AChE staining. CCL: cortical cell layers; DG: dentate gyrus; M: molecular layer; G: granular layer. Magnification – (cortex: 200X), (Hippocampus: 300X), (cerebellum: 600X).

Discussion

The results of the present study clearly demonstrate a significant inhibition in AChE activity by Pb in a dose and age dependent manner and recovery from the Pb-induced alterations in AChE activity and expression with the supplementation of Ca^{2+} or Zn^{2+} in mouse brain. AChE is an enzyme that terminates the signal transmission at the cholinergic synapses of neurons by rapid hydrolysis of the neurotransmitter ACh. Generally brain has the highest AChE activity than other tissues. The different regions of brain are known to perform different functions and any change in the cholinergic system in these sections will reflect in the performance. In general, during development, an increase in total AChE

activity can be observed in the central nervous system (2, 24, 37). In mouse brain, total AChE activity increases about 15 fold between embryonic day (ED) 9 to ED 19. This developmental change is characterized by a continuous increase in the amphiphilic tetrameric (G4). AChE form a transient increment in the amphiphilic monomer (G1) form which reaches a maximum by ED 17, and a gradual increase in both hydrophilic G1 and G4 forms through to ED 19 (23). The active site of AChE comprises two subsites critical for its functioning. One of these has traditionally been termed the anionic subsite because it was hypothesized to consist of one or more negatively charged groups that electrostatically interacted with the positively charged quaternary nitrogen (N) of the substrate. The other region of the active site is the so called esteratic subsite, which possesses a serine residue that is responsible for driving the hydrolytic reaction (17, 33, 55).

The results of the present study showed inhibitory action of Pb on AChE activity in all the four brain regions; cortex, hippocampus, cerebellum and medulla. It is noteworthy that the alterations in AChE activity were continued even after the Pb-exposure was with drawn. Heavy metal ions exert a potent inhibitory effect on different enzymes such as AChE by binding avidly to sulfhydryl (–SH) groups with similar affinities (7, 10). Pb has high affinity for the free sulfhydryl groups in enzymes and proteins and its binding can alter their correct function. The effect of Pb is observed to be more in the fetal brain due to the immature BBB (blood brain barrier) and the absence of protein complexes that sequester metals in mature tissues (4). The decrease in AChE activity in young mice could be related to the fact that Pb crosses BBB quite readily (27). This may be the reason for higher inhibition of AChE in the brain tissue of young mice as compared to three month old mice. Highest inhibition on PND 28 may be due to longer Pb-exposure time. The inhibition of AChE could also be due to the damage of the brain cells. Pb-toxicity may have induced the above conditions in mouse brain tissues and hence a decrease in AChE activity was observed. Pb disturbs the normal development of the brain, causing reductions in cellular development in the cerebellum (46), cerebral cortex (41) and hippocampus (11).

A review of previous reports suggests the possibility of functional association between the effects of early Pb-exposure, hippocampal damage and cholinergic deficiency (1). Pb-exposure produces a direct effect on AChE activity in the developing cerebellum leading to alterations in motor coordination (20). The susceptibilities of different brain areas to Pb-exposure could be related to local differences in their formation and maturation. The cholinergic synapses were more in hippocampus, followed by cortex, cerebellum and medulla. This may be the reason for higher AChE activity observed in hippocampal region as compared to cortex, cerebellum and medulla. It is known that the differential sensitivity to Pb neurotoxicity in the different brain regions is not due to a preferential Pb accumulation, but is possibly due to alterations in the biochemical or cellular processes that are uniquely associated with, or greatly enhanced in a particular region (36, 59).

The inhibition of AChE following Pb-exposure appeared to comprise a correlation between increases in the lipid peroxidation observed in our study (42). An inverse correlation between AChE and lipid peroxidation in brain has been reported by Sandhir et al., (49). Pb may also exert its neurotoxic effects via peroxidative damage to the membranes.

AChE activity inhibition was observed following chronic exposure of Pb to the young mice through their mother's milk. The sensitivity of

immature brain to the neurotoxic effect of Pb and the increased resistance in the adult brain may be due to its defense mechanisms developing with age (62). The gradual recovery of AChE activity in three month mice can be attributed either to spontaneous reactivation of the enzyme or the denovo synthesis of the enzyme molecule or both (14). It is also dependent on the degree of exposure and its initial depression (39). The high dose of Pb-exposure resulted in higher inhibition of AChE activity. This may be due to more binding of Pb to -SH groups in addition to more accumulation of Pb in various regions of brain tissue.

The dentate gyrus and CA1 sub regions of the hippocampus have distinct developmental profiles, CA1 developing primarily prenatally in rodents, the dentate gyrus maturing much later in the postnatal period (6). Pb impaired LTP in area CA1 and dentate gyrus coincided with impaired spatial learning in the water maze (45). Van der Zee et al., (57, 58) established a learning related increase in immunoreactivity to the gamma isoform of protein kinase C (PKC) in hippocampal soma and dendrites. They found learning-related increase in immunoreactivity for muscarinic acetylcholine receptors in hippocampal pyramidal cells of area CA1-CA2 and a learning related decrease in the immunoreactivity of the non pyramidal neurons of the same region. The decrease of AChE activity with Pb-exposure can be correlated with the expression of AChE with histochemical staining in this study. The intense staining appeared in control brain and significantly loss of staining was noticed in Pb-exposed brain especially in CA3 and DG region of hippocampus, cortical cell layers, and molecular granular cell layers of cerebellum.

Supplementation with Ca^{2+}/ Zn^{2+} reversed the Pb-induced inhibition in AChE activity and also the loss of AChE staining in different brain regions. However, the Ca^{2+} supplemented brain regions did not show significant loss of AChE staining confirming the protective effect of Ca^{2+}. The reversal of inhibition in the activity of AChE by supplementation with Ca^{2+}/ Zn^{2+} may be due to competition of these metals for similar binding sites and reducing the availability of binding sites for Pb. Supplementation with Ca^{2+}/ Zn^{2+} reduces the gastrointestinal absorption of Pb and decreases Pb retention.

Ca^{2+} is a divalent cation just like Pb. Because the same transport mechanism is operative for absorption of Pb and Ca^{2+} from the gastrointestinal tract there is resulting competitive interaction between Pb and Ca^{2+} (38). Studies have shown that Pb has an inhibitory effect on the peripheral nervous system through stimulus coupled or Ca^{2+} dependent release of acetylcholine . Absorption of Pb by gastrointestinal tract is inversely related to the amount of Ca^{2+} present (21, 31). Furthermore Ca^{2+} supplements had a protective effect by significantly reducing blood Pb levels in pregnant women whose diets were deficient in Ca^{2+} (16). Ziegler et al., (61) observed an inverse relationship between dietary Ca^{2+} and Pb retention and absorption in young infants. Ca^{2+} and Pb compete for similar binding sites on intestinal mucosal proteins, which are important in the absorptive process (19). These shared binding sites on absorptive proteins would explain why sufficient Ca^{2+} decreases Pb absorption. The achievement of adequate rather than excessive dietary Ca^{2+} seems to be more useful in combating Pb intoxication (40).

Zn^{2+} counteracts the inhibitory action of Pb in animals (28). Zn^{2+} deficiency reduces the activity of numerous enzymes in the brain that may lead to a stop in cell multiplication at a crucial period in morphogenesis (3, 53). Potential mechanisms of interaction between Pb and Zn^{2+} include an inhibition of Pb gastrointestinal absorption by Zn^{2+}, which has been demonstrated

in rats (9). Indirect evidence includes the observation that supplemental Zn^{2+} decreased the concentrations of Pb in blood and in a variety of tissues, in Pb-exposed mice. Thus supplementation with Ca^{2+} or Zn^{2+} reduced the inhibitory effect of Pb on the activity of AChE enzyme and reversed the enzyme activity nearer to control level.

The changes produced in the levels of AChE during Pb treatment indicate the degree of neuronal impairment which in turn produces functional and structural damage to the tissue depending on the dose. The supplementation with Ca^{2+}/Zn^{2+} reduced the Pb-induced alterations in AChE activity, with Ca^{2+} supplementation being more protective as compared to Zn^{2+}.

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Isolation of secondary products from *Ipomoea digitata* – a medicinally important plant

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Abstract

The paper reports the isolation and characterization of β-sitosterol, t-cinnamic acid [undecyl (E)-3-(4- hydroxyphenyl)-2-propenoate], an unknown coumarin and a lignan type resin glycoside from the tuberous roots of *Ipomoea digitata*. The structures of the compounds were elucidated on the basis of extensive chemical and spectroscopic data. Importantly, one of the compounds exhibited significant antibacterial activity against *Pseudomonas aeruginosa* and *E coli*. The resin glycosides are known as purgative ingredients and hence have medicinal value. However, the exact chemical and biological activity of the resin glycoside isolated in this study is yet to be ascertained.

Key words: *Ipomoea digitata*, Convolvulaceae, Undcyl (E)-3-(4-hydroxyhenyl)-2-propenoate, Coumarin, Resin glycoside

Introduction

Ipomoea digitata is a wide-spread, paleotropic, perennial herb belonging to the family Convolvulaceae. It is commonly known as wild yam or Vidari Kanda in Telugu. The tuberous roots are used as tonic, aphrodisiac, cholagogue, demulcent, diuretic, emmenagogue, galactogogue and rejuvenant. Aqueous infusions of the roots are used in Indian traditional medicine for treating epileptic seizures and as antioxidative in Ayurvedic medicine. Hundred grams of Dabur Chavanprash contains 1.195 g of *I. digitata* root powder. Studies on some other species of this genus revealed the presence of Ipangulins, the first pyrollizidine alkaloids, ergoline alkaloids, stoleniferins and resin glycosides (1-6). Recent reports revealed the presence of resin glycosides in the leaves and stems of *I. digitata* (7). The aerial parts are also used as folk medicine in Japan (8). Chemical constituents of the root of this plant (scoperon, β-sitosterol, taraxerol) were reported earlier by Rao et al. (9). The pharmacological activity of the fraction D (10) of this plant extract has drawn our attention to the chemical and biological study of this plant. One of our efforts to discover the structurally diverse and biologically significant metabolites from *I. digitata* has led to the isolation of two known (named as compounds 1, 2) and two unknown compounds (compounds 3 and 4) with spectral and chemical data. We also report, antibiotic activity of compound 3 on gram-negative bacteria *Pseudomonas* and *E. coli.*

Materials and Methods

Fresh tuberous roots of *Ipomoea digitata* were collected from Nallamala forests of Andhra Pradesh, India. The plant specimen was deposited in the herbarium of the Department of Botany,

Osmania University, Hyderabad, India. Three kilograms of fresh tuberous roots were cut into small pieces and dried under shade. The dried tuberous roots were pulverized by using a mixer and extracted in hexane by dipping it for 3 days, followed by re-extraction in methanol. The hexane extract was filtered and concentrated under reduced pressure. The methanol extract gave 83 g of residue after concentration under reduced pressure. With the aim of isolating polar compounds, the dried root powder (1 kg) was taken freshly and extracted in water for 3 days. Hexane extract was subjected to silica gel column chromatography, eluted with a solvent system of hexane/ethyl acetate to separate the compounds from the mixture. The fraction (10 g) eluted by hexane and ethyl acetate (4:96) resulted in the isolation of a pure compound (compound 1) as determined by thin layer chromatography (TLC). This compound was further analyzed by nuclear magnetic resonance (^{1}H NMR) and mass spectrometry (MS) for identification. The fraction eluted by hexane : ethyl acetate (95:5) was repeatedly subjected to silica gel purification which afforded 65 mg of the compound. The white powder was successively subjected to ^{1}H NMR, MS, infra red (IR) and ^{13}C NMR analysis for structure determination (compound 2). The fraction (28 g) of methanolic extract, eluted with chloroform : ethyl acetate (80:20), gave compound 3. Upon extracting this in hexane and benzene (50:50) for 12 h, yellow coloured needles were obtained. The compound was then subjected to ^{1}H NMR, MS, IR and ^{13}C NMR. The dried water extract was concentrated and extracted again in chloroform (2 x 500 ml) to remove low polar compounds. The remaining residue dissolved in methanol consisted of a single compound that was confirmed by TLC. The methanolic extract (1.5 g) was dried and concentrated to give a semi-solid sticky material, which was separated by column chromatography on silica gel (100:200 mesh). This was separated with chloroform : methanol (50:50) solvent system in the column and designated as compound 4.

The IR spectra were recorded on a Bruker Tensor 27 FT- IR spectrometer with KBr pellets. The ^{1}H NMR spectra were recorded at 400 MHz (Varian make). The ^{13}C NMR spectra were performed in $CDCl_3$ at 75.5 MHz for compounds 1 and 2 but ^{13}C NMR of compound 3 was noticed in dimethyl sulphoxide (DMSO) at 75.5 MHz. Electron ionization-MS were obtained at 70 ev and fast atom bombardment (FAB)–MS using Argon (6 kv) as the FAB gas. Silica gel (60-120 mesh and 100-200 mesh, Merck, Darmstadt, Germany) was employed for column chromatography. TLC was carried out on pre-coated kiesel gel 60 F_{254} (0.25 thick, Merck, Darrnstadt, Germany) plates, with hexane-ethylacetate and chloroform–ethylacetae as solvent systems. Coloured spots were visualized by exposure to iodine vapours followed by spraying with 10% sulfuric acid solution. Dragendorff"s reagent was used for the detection of alkaloids. High performance liquid chromatography (HPLC, Eclipse XDB-C-18 column, 5 µm, 4.6 x150 mm, 25%-100% methanol in water over 8 min followed by 100% methanol to 11 min, 1 ml/min, 30 ^{0}C) was carried out in combination with TLC. Methanol, chloroform, ethyl acetate and hexane which were used for extraction of compounds were purchased from the Finar chemicals, Hyderabad. Methanol-sulfuric acid, Dragendorff reagent, ethanol-sulfuric acid were used as reagents to detect the compounds that are separated on the chromatogram. Silica coated glass plates for TLC were purchased from Merck. All other reagents and chemicals used were of analytical grade.

Antibacterial activity was tested against Gram negative bacteria like *Pseudomonas* and *E.coli* by single disc method (11). Test bacterial

strains were cultured in sterilized Luria Bertani (LB) broth (pH 5.7) for 16-18 h at 37 ^{0}C on a rotary shaker. LB media with agar as gelling agent was prepared and sterilized at 121 ^{0}C for 15-20 minutes in an autoclave. Plating was carried out under aseptic conditions. A loop full of liquid broth of bacterial culture was spread over solidified LB media. Sterilized discs (Whattmann filter paper) were placed at equal distances. The four compounds each of 5 mg were weighed and dissolved in 5 ml of DMSO. Dissolved compounds (3 µl) were placed on the discs and plates were wrapped tightly with parafilm and incubated at 37 ^{0}C for 24 h.

Results and Discussion

Extraction of tuberous root powder of *Ipomoea digitata* resulted in the isolation of four compounds. The first compound is a smooth, whitish pink, amorphous powder. The structure (Fig. 1) of this compound (β-sitosterol) was determined based on the spectral data like ^{1}H NMR and Mass (Fig. 2) and also correlated with the previously available data (12). Compound 2, isolated for the first time from *I. digitata*, is a white powder, with a melting point of 80 ^{o}C, chemical formula of $C_{20}H_{30}O_3$ and is identified as undecyl-(E)-3-(4-hydroxyphenyl)-2-propenoate (trans-cinnamic acid). Its structure and ^{13}C NMR spectrum are shown in figures 3 and 4 respectively. The third compound appeared as a pale yellow coloured needles with a melting point of 205 ^{o}C and identified as 5-hydroxy-7-methoxy coumarin based on its spectroscopic data. Its structure and ^{1}H NMR spectrum are shown in figures 5 and 6. It exhibited the activity under ultraviolet light. Compound 4 is soluble only in methanol and water, and is a brown coloured, sticky, semi solid, resin type glycoside. The signals in ^{1}H NMR (Fig. 7) and Mass spectra allowed us to conclude that this compound is a resin glycoside. The ^{1}H NMR and Mass spectral signals of compound 4 did not show any correlation with the available spectral data (7), indicating that this compound is different from the compounds already isolated from the leaves and stems of *I. digitata* earlier. However, the exact physical nature and chemical structure of the glycoside is yet to be determined.

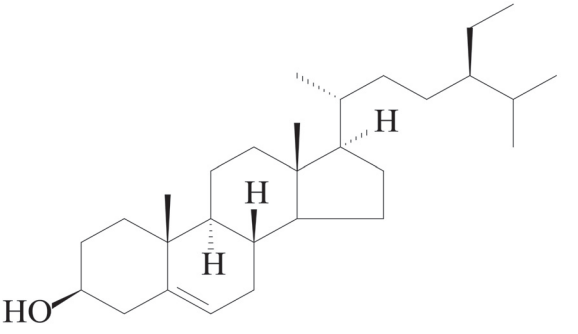

Fig. 1. Structure of β-sitosterol

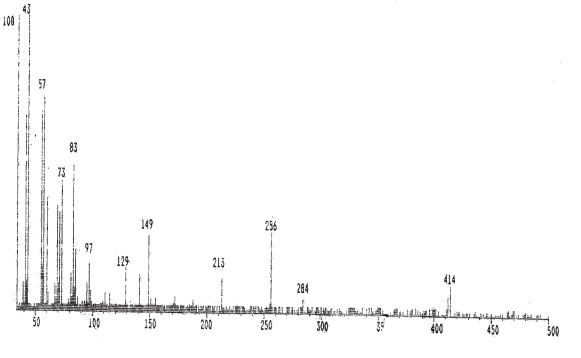

Fig. 2. Mass spectrum of β-sitosterol

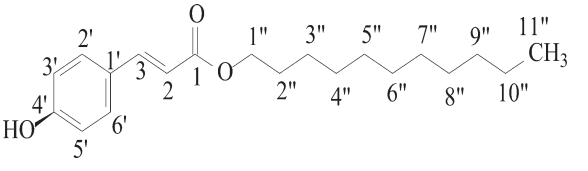

Fig. 3. Structure of t-cinnamic acid (undecyl (E)-3-(4-hydroxyphenyl)-2-propenoate)

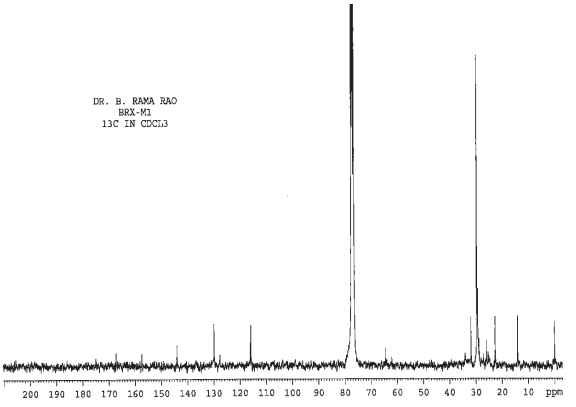

Fig. 4. ^{13}C NMR spectrum of t-cinnamic acid

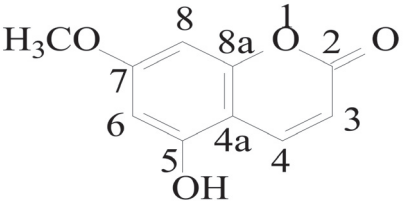

Fig. 5. Structure of 5-hydroxy-7-methoxy coumarin

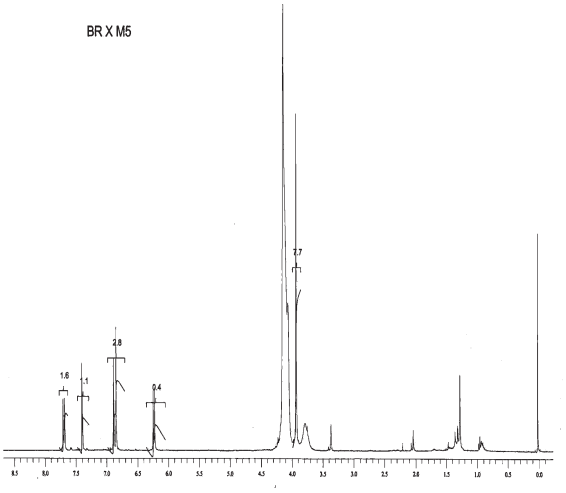

Fig. 6. ^{1}H NMR of 5-hydroxy-7-methoxy coumarin

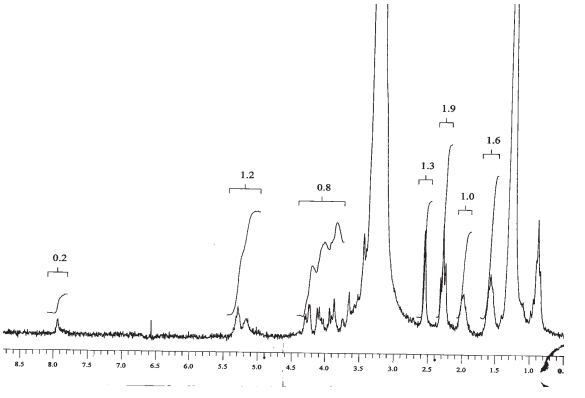

Fig. 7. ^{1}H NMR spectrum of lignan type resin glycoside

None of the above compounds showed any antioxidant activity though extracts of this plant are used in Ayurveda as an antioxidant. However, coumarin, when tested against *Pseudomonas aeuriginosa* and *E. coli*, inhibited the growth of these bacteria but not others. This is the first time that bacterial activity has been found for the coumarin isolated from this plant. Hence, *I. digitata* can serve as a good source for the isolation of this bioactive compound. The inhibition zone (14 mm) of bacterial growth was observed around the disc containing the compound. Many workers reported major effects like antinociceptive, antifungal and anticancer activities for the compounds of *Ipomoea* genus (13-15). The resin glycosides, originated from the Convolvulaceous plants, are well known as the purgative ingredients in some traditional medicines. They are divided into ether-soluble resin glycosides "jalapin" and ether-insoluble resin glycoside "convolvulin" (16). Resin glycosides have also recently been isolated and characterized from the leaves and stems of *I. digitata* (7). They named it as digitatajalapin I since it was isolated from jalapin fraction. The resin glycoside isolated in the present study is ether-soluble type. Therefore, it could be a "jalapin" related glycoside. However, the biological effects of the resin

glycoside isolated in this study needs to be determined, which is underway.

Acknowledgement

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Measurement of IgA activity against parasitic larvae, fecal egg count and growth rate in naturally infected sheep

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Abstract

Gastrointestinal parasitism is one of the greatest causes of disease and lost productivity in domestic animals. Anthelmintic treatment is the mainstay of current control methods. However, with the widespread use of anthelmintics, the problem of parasite resistance has emerged. Other modalities of prevention and treatment are urgently needed. One of the most promising developments in this respect is the use of genetically resistant sheep. Selective breeding of animals resistant to gastrointestinal parasitism is particularly attractive, but identifying the phenotypic and genetic markers of resistance on which selection will be based is a major problem. Hence, a study was undertaken to investigate some of the phenotypic determinants of resistance to nematode infection in Scottish Blackface sheep when naturally infected with the gastrointestinal nematodes, particularly *Teladorsagia circumcincta*, and how these determinants may facilitate the successful selection of resistant animals. The present study has not found any correlations between plasma IgA activity against third-stage larvae of *T. circumcincta* and fecal egg. Moreover, there was no significant correlation between growth rates in 24 week old lambs. The results suggest that IgA activity against fourth-stage larvae is probably a better marker of nematode resistance than IgA activity against third-stage larvae. The work detailed here has further increased our understanding of the complex host/parasite relationship, and has confirmed that selective breeding, using the various phenotypic markers.

Key words: IgA activity, *Teladorsagia circumcincta,* Host-Parasite relationship

Introduction

Gastrointestinal nematode parasitism is arguably the most serious constraint affecting sheep production worldwide (6). Of the novel approaches to parasite control, breeding sheep for resistance seems to be the most promising application that can be used to complement the strategic use of dewormers and improved pasture management (2). Among gastrointestinal nematode infection, *Teladorsagia circumcincta* is important in Scottish Blackface sheep. *T. circumcincta* has a direct life cycle (no intermediate host) and involves development through a series of cuticle shedding moults. There are three post-hatching pre-parasitic stages L_1, L_2 and L_3 and two parasitic stages L_4 and a final L_5 immature adult stage. Hatched larvae become infective (L_3 larvae) in about 6 to 7 days and most larvae survive on pasture for 4 months, although some larvae may survive much longer. Infection is by ingestion of L_3 larvae by the host while grazing. The L_3 larvae penetrate the lumen of the abomasal gland and moult to the L_4 stage. Following

a period of growth and a further moult to the L_5 (immature adult) stage, they emerge from the gland and mature on the mucosal surface. Male and female worms copulate producing eggs, which are passed by the host in the faeces. The eggs hatch to the L_1 stage and further development to the L_2 and L_3 stages on pasture. Typically this life cycle takes three weeks to complete with variation depending on the weather and immune status of the animal (14). Anthelmintics have a strong effect in limiting worm burdens, and in particular in controling adult worms. However, eggs can be found in faeces within 14-28 days after drug treatment coming from fresh infections or from worms that survive treatment. The development of resistance to anthelmintics is a major threat to parasite control worldwide (15).

Nematodes cause disease but perhaps their major economic impact is the reduction in growth of young lamps. Due to the emergence of anthelmintic resistance in parasite population, non-chemotherapeutic methods are being investigated to control *T. circumcincta* infection (1). A detailed understanding of the immune emergence of anthelmintic resistance in parasite populations, mechanism involved in resistance to infection will lead to more sustainable methods of control and in particular will aid in the identification of resistant animals.

Fecal egg count has been widely used as an indicator of host resistance to gastrointestinal parasites in sheep and has been shown to be a heritable trait (7). The strength of the association between IgA and parasite fecundity led to hypothesis that the specificity and activity of local IgA was the major mechanism regulating the fecundity of *T. circumcincta* (11) and a major mechanism of resistance to infection in lambs. A comparison of Ig A response to third stage, fourth stage and adult *T. circumcincta* indicated the strongest association with reduced worm length, with increased response to fourth stage larvae (12). The response of the third stage larvae were correlated with the response to fourth stage larvae (10). IgA activity against fourth stage larvae probably is the best phenotypic marker, but recovering fourth stage larvae requires killing sheep. Third stage larvae can be recovered from fecal cultures, making responses to third stage larvae cheaper and easier to measure and killing animals can be avoided. There appear to be very few studies that have examined IgA activity against third stage larvae as a phenotypic marker for resistance in naturally infected sheep.

The present investigation was undertaken to determine the relationships between IgA activities against third stage larvae of *T.circumcincta*, fecal egg counts and growth rate of Scottish Blackface lambs and to explore whether plasma IgA activity against third stage larvae could be used as indicator trait for resistance or susceptibility in sheep.

Materials and Methods

Animals: Seven hundred and fifty nine naturally infected six month old Scottish Blackface lambs were sampled for blood and feces in October for a period of three years.

***Fecal Worm Egg Count*:** Feces samples were taken directly from the rectum of the lambs and stored at 4°C until processed. A modified McMaster salt flotation technique (5, 16) was performed to estimate the concentration of nematode eggs in the feces.

***Weight of the lambs*:** Individual body weight of the experimental lambs was recorded using a sheep weighing scale at each blood sampling and fecal collection dates.

Preparation of third stage larvae: Third stage larvae were collected from fecal cultures of eggs of deliberately infected sheep and exsheathed in 1% Sodium hydrochloride in phos-

phate buffered saline (PBS, pH 7.4) for 10 minutes at 37°C. Exsheathed larvae were then resuspended in 50 ml PBS and centrifuged at 100g for 10 minutes. The supernatant was removed and then washed twice. The larval pellet was washed once in PBS containing 100 IU ml^{-1} penicillin, 0.1 mg ml^{-1} streptomycin, 2.5 µ g ml^{-1} amphotericin B and 0.05 mg ml^{-1} gentamicin to prevent any contamination (10). Larvae were then given a final wash in 50 ml of 10 mM Tris buffer (pH 8.3), containing 1mM disodium ethylene diamine tetracetic acid (EDTA), 1mm ethylene glycol bis (2-aminuteso ethyl ether)-N,N,N'N-tetracetic acid (EGTA), 1mM N-ethylmaleimide (NEM), 0.1 µ g pepstatin, 1mM phenyl methyl sulphonyl fluoride (PMSF), and 0.1 mM N-tosylamide-L-phenylalanine chlormethyl ketone (TPCK) as protease- inhibitor solution containing 1 % sodium deoxycholate and homogenized using a handled electric homogenizer on ice. When larvae were completely homogenized, they were centrifuged at 500g for 20 minutes and the soluble extract was filtered through a sterile 0.2µm syringe filter. The extract was again spun at 500g for 20 minutes and the supernatant aliquoted and stored at -80°C.

***Blood Samples*:** Blood samples were collected by jugular venepuncture into evacuated glass tubes containing 20mM disodium EDTA as an anticoagulant. Plasma and buffy coats were obtained by centrifugation at 100g for 30 minutes and stored at -20°C for further use.

Enzyme-Linked Immunosorbent Assay (ELISA): ELISA was used to detect Parasite specific host plasma IgA activities to infection against somatic larval extracts of third stage larvae from *T. circumcincta*. Each well of a 96-well flat-bottomed microtitre plate (Nunc) was coated with 100 µl of larval antigen preparation (L_3) at 5µg ml^{-1} in 0.06M sodium carbonate buffer pH 9.6 overnight at 4°C. The plates were then washed five times in PBS-T. The individual sheep plasma samples were diluted 1:10 in PBS + 0.4% skimmed milk (PBS-TSM). The plates were then incubated with 100 µl per well, in duplicate for 30 minutes at 37°C. Positive and negative controls were diluted 1:10 in PBS-TSM and run in triplicate on each plate to minimize the effect of variation between plates on different days. Plates were incubated with 100 µl per well of a monoclonal rat IgG anti-sheep IgA diluted in PBS-TSM for 30 minutes at 37°C. After five washes in PBS-T, the plates were incubated with 100 µl per well of a mouse anti-rat IgG alkaline phosphatase antibody conjugate (Sigma) diluted in PBS-TSM for 30 minutes at 37°C. After a final five washes in PBS-T, the plates were incubated in 100 µl per well with Bluephos® Microwell Phosphatase Substrate system (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) at 37°C. The optical density of each well was then read at 635 nm with a multichannel spectrophotometer (Titertek Multiscan MC, Labsystems, Oy, Finland or Dynex MRX, Dynex Technologies, Ashford, UK) for 15 minutes. Usually this was done at five minute intervals, until the positive control optical density reading was between 1.5-2. Each batch of monoclonal and secondary antibody was titrated to determine the appropriate concentration. This procedure was carried out on the Grifols Triturus® ETA Analyser.

***Optical density indices*:** The Positive and negative controls in each ELISA assay were pooled plasma samples from individual animals which had given either very strong (positive control) or very weak (negative control) optical densities using the methods of Sinski et al. (10). To minimize the variation between results obtained on different days and between plates, optical densities for each sample were transformed into an optical density (OD) index (10).

Statistical Analysis: The association between IgA activity, fecal egg counts and growth rate was estimated by correlation coefficient using the correlation procedure in the SAS statistical package (SAS Institute, Cary, NC, USA). Egg counts were transformed prior to statistical analysis by taking the logarithm of the egg count plus 10.

Results

IgA activity against an extract of third-stage larvae was measured using an indirect ELISA and expressed as a percentage of a standard value. Fig. 1 shows the regression between replicate measurements of IgA activity in October during the first year. The two measurements were carried out to evaluate the repeatability between ELISAs with the same samples on different dates. The regression shows that the two measurements gave similar results. The correlation between the first and the second run was positive and very highly significant ($r = 0.80$, $p < 0.001$).

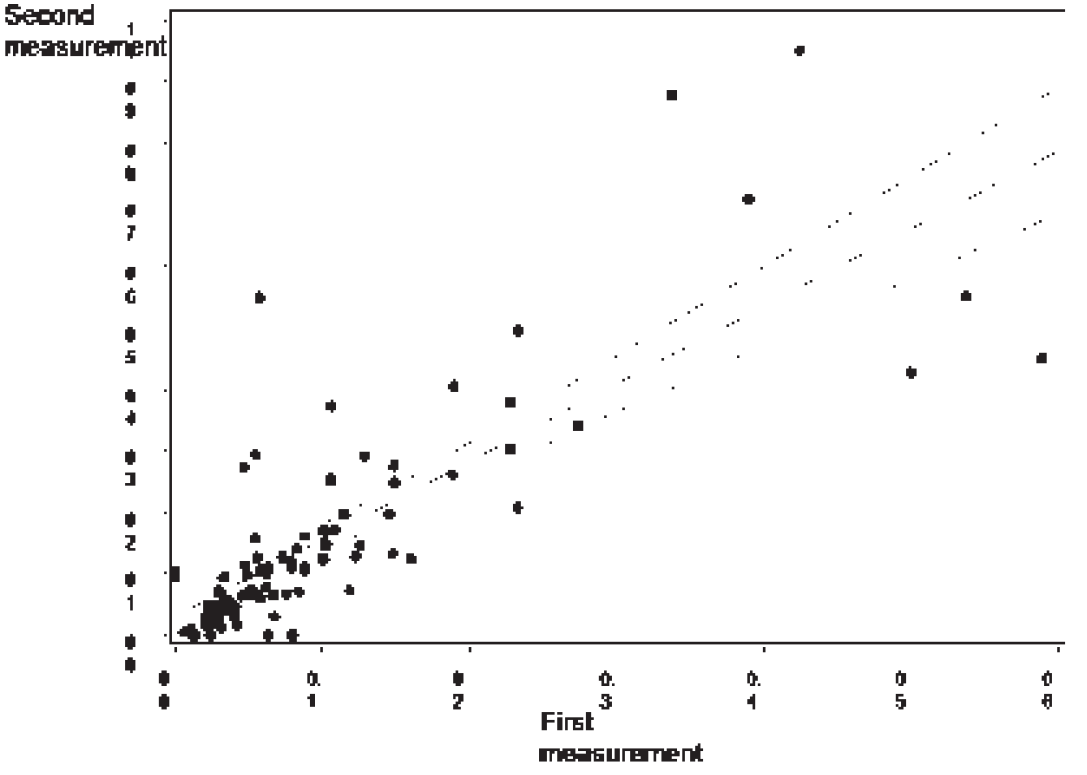

Fig.1 Regression between replicate measurements of IgA activity against third-stage larvae.

The distribution of IgA activities against third-stage larvae was positively skewed in each year; most lambs had relatively low values but some lambs had quite high values. Fig.2 shows the distribution of IgA activity against third-stage larvae of *T. circumcincta* as measured by simple indirect ELISA in the lambs sampled in October during the three years of study.

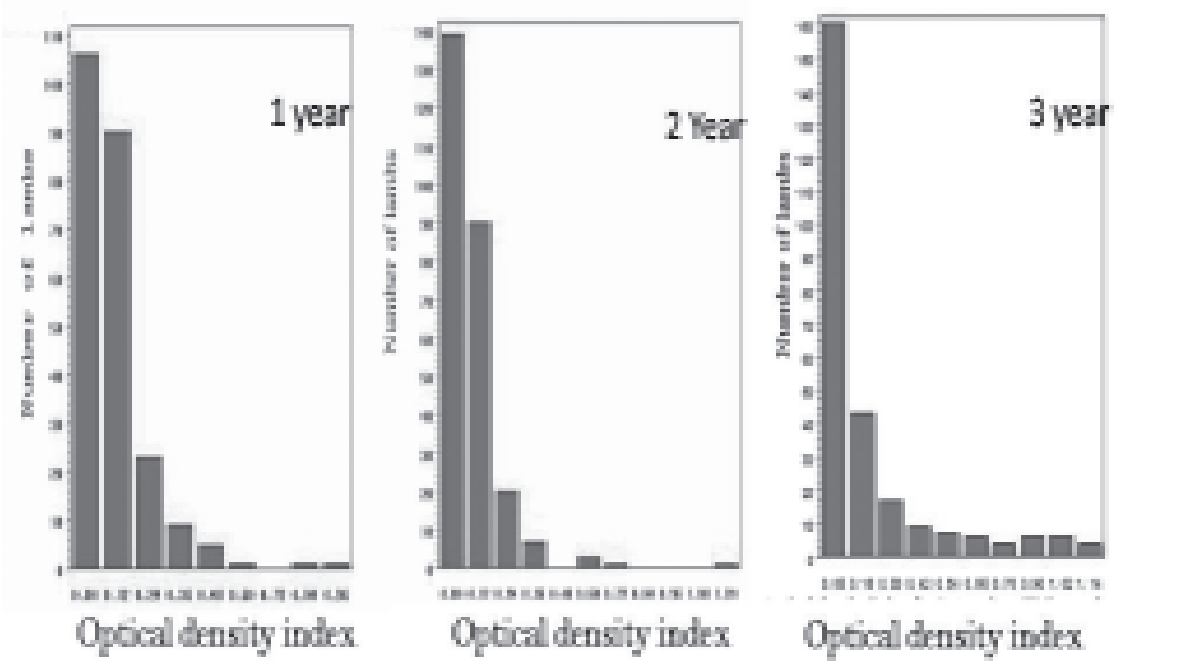

Fig. 2 Distribution of plasma IgA activity against third stage larvae of *T. circumcincta* measured by Simple Indirect ELISA in naturally infected lambs during the period of study

The number of animals, mean IgA activity, standard error of mean, minimum and maximum value observed in October during the period of study are given in Table I. The mean plasma IgA activity was 0.11 in first year, 0.09 in second year and 0.19 in third year. Mean IgA activity varied among years but the standard errors are quite small. The mean IgA optical density indices ranged from 0-1.02 in the first year, 0-1.24 in the second year and 0-1.12 in the third year.

Table 1. Mean and standard error of IgA activity against third-stage larvae of *T. circumcincta*

Date of sampling	Number of animals	Mean of IgA activity	Std. Error of mean	Minimum	Maximum
1year-October	236	0.108	0.008	0.000	1.02
2year-October	261	0.091	0.007	0.000	1.24
3year-October	262	0.186	0.000	0.000	1.12

Table 2. Eggs per gram of feces in lambs sampled during three years

Date of sampling	Mean	Std. Error of Mean	Minimum	Maximum
1 year October	278.2	19.1	0	1700
2 year October	181.6	13.4	0	1200
3 year October	252.7	14.5	0	1238

The mean faecal egg count was 278.2, 181.6 and 252.7 respectively during the three years studied (Table 2).

The Pearson correlation coefficients were calculated from log-transformed fecal egg counts within each year in the 1st year (r = 0.014, p = 0.826), 2nd year (r = - 0.091, p = 0.147) and 3rd year (r = 0.067, p = 0.286) against the log. transformed optical density indices of IgA activity(Table 3). The results confirm that there was no significant correlation between fecal egg counts and IgA activity against third-stage larvae.

Tble 3. Correlations between transformed fecal egg counts and IgA activity

	Log epg 1st Year	Log epg 2nd year	Log epg 3rd Year
IgA activity	0.014	-0.091	0.067

Further, there was no significant correlation between IgA activity and growth rate at 24 weeks old lambs for the 1st years (r = - 0.079, p = 0.248), 2nd year(r = - 0.143, p = 0.021) and 3rd year (r = - 0.042, p = 0.503)(Table 4).

Tale 4. Correlations between growth rate at 24 weeks old lambs and IgA activity

	weight in 1st Year	weight 2ndYear	weight in 3rd Year
IgA activity	-0.079	-0.143	-0.042

Discussion

Plasma IgA activity was measured against third-stage larvae of *T. circumcincta* in naturally infected sheep, using a simple, indirect ELISA. The results show that the distribution of plasma IgA activity against third-stage larvae in all lambs was positively skewed with the majority of lambs having relatively low activity but a minority lambs had quite high activity (Fig 2). The distribution is similar to the distribution of IgA activity against fourth-stage larvae of *T. circumcincta* (13). Serum IgA is dimeric in ruminants and in experimental studies almost totally derived from the gastrointestinal tract (9). The function of IgA has

been thought to be restricted to binding antigens outside the epithelium basal membrane (14).These results suggest that plasma IgA activity against third-stage larvae may provide a window on local IgA responses in sheep.

No attempt has been made to quantify the amount of IgA in plasma samples. The optical density depends upon the amount of IgA present and the avidity and affinity of IgA for the component of the antigen preparation. As the antigen preparations were complex mixtures, any attempt to estimate absolute antibody concentrations would have been tedious and prone to error (10). The mean optical density indices of plasma IgA activity did not show large differences among the values in the three years.

IgA activity in the serum was dependent upon IgA activity in the abomasum and also the number of adult nematodes present in the abomasums (10). During the early phase of the infection parasites suppressed the acute phase response and the complement system of the host (8)**.**In addition, there was a strong positive relationship between responses to third-stage and to fourth-stage larvae. The correlation coefficients ranged from 0.60 to 0.79 ($P < 0.001$) for parasite-specific IgA, which showed that sheep with strong responses to third-stage larvae tended to have stronger responses to fourth-stage larvae. Correlated results for the same animals could be due to the existence of some shared, or similar, antigens in the different larval stages. Correlations between responses do not necessarily imply similar amounts of antibody. Positive correlations merely imply that animals that gave higher-than-average responses in the first test gave higher-than-average responses in the second test. So far little work has been done to measure the plasma IgA activity against third-stage larvae of *T. circumcincta* in naturally infected lambs. . The present result suggests that IgA activity against third-stage larvae as a possible marker of resistance to nematode infection in sheep could be used rather than forth-stage larvae but more research is needed. The experimental results did not reveal any correlations between fecal egg counts or IgA activity against third-stage larvae. In contrast animals with increased IgA activity against fourth-stage larvae had lower faecal egg counts (13). Therefore IgA activity against fourth-stage larvae is probably a better marker of nematode resistance than IgA activity against third-stage larvae

Other results reported weak correlations between IgA responses to third ($r = - 0.155$) and fourth-stage ($r = -0.176$) larval extracts and egg counts (3) . The reasons for the differences are unknown but may be due to differences in exposure to nematode infection or nutrition. Unfortunately the results came from lambs on commercial farms and could not be investigated further. However, further trials considering different breeds and age groups of lambs at different periods of years at different environmental conditions should be considered. There were no significant correlations between weight gain at 24 weeks old lambs and IgA activity during the years studied. There appear to be no previous published studies that have reported the relationship between IgA activity and growth rate. More research is necessary to examine the relationship between nematode resistance and IgA activity against third-stage larvae. Further experiment would require necropsy of large numbers of naturally infected animals.

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Development of bilayered mucoadhesive patches for buccal delivery of felodipine: *in vitro* and *ex vivo* characterization

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Abstract

Bilayered buccoadhesive patch for systemic administration of felodipine was developed using hydroxy propyl methyl cellulose as primary layer and Eudragit RLPO as secondary layer. *In vitro* drug permeation studies through porcine buccal membrane and buccal absorption studies in human volunteers were performed. Six formulations were developed by solvent casting technique and evaluated for *in vitro* drug release, moisture absorption, mechanical properties, surface pH, *in vitro* bioadhesion and *in vitro* permeation of felodipine through porcine buccal membrane from bilayered buccal patch. Formulation BB4 showed a drug release of 94.6 % with zero order release profile and permeated 41.6 % of drug with a flux of 0.113 mg $h^{-1}cm^{-2}$ through porcine buccal membrane. Formulation BB4 showed 3.42 N and 1.63 mJ peak detachment force and work of adhesion respectively. The physicochemical interactions between felodipine and polymer was investigated by Fourier transform infrared (FTIR) Spectroscopy. According to FTIR the drug did not show any evidence of an interaction with the polymer. A stability study of optimized patch BB4 was done in natural human saliva; it was found that both drug and buccal patches were stable in human saliva. The results indicate that suitable bilayered buccoadhesive patches with desired permeability could be prepared.

Keywords: Buccal patches, felodipine, bioadhesion, mechanical properties, buccal delivery

Introduction

Buccal delivery of drugs provides an attractive alternate to the oral route of drug administration, particularly in overcoming deficiencies associated with the oral route. Buccal mucosa has an excellent accessibility, an expanse of smooth muscle and relatively immobile mucosa, hence suitable for administration of retentive dosage forms. The direct entry of the drug into the systemic circulation avoids first-pass hepatic metabolism leading to increase in bioavailability (1, 2). Other advantages such as low enzymatic activity, painless administration, easy drug withdrawal, facility to include permeation enhancers/enzyme inhibitors or pH modifiers in the formulation and versatility in designing as multidirectional or unidirectional release systems for local or systemic actions (3). Various mucoadhesive formulations were suggested for buccal delivery that includes buccal patches (4, 5), adhesive tablets (6, 7) and adhesive gels (8). However, buccal films are preferred over adhesive tablets in terms of flexibility and comfort (9).

Felodipine (FDP), a calcium channel blocker belonging to dihydropyridines is used as a potent

peripheral vasodilator, which effectively reduces blood pressure when given at doses of 5–20 mg per day. After a single, 20 mg oral dose of FDP, peak plasma concentrations are achieved within 2.5–5 hours (10). It was reported to be well absorbed following oral administration, but undergoes extensive first pass metabolism; leading to poor bioavailability (11). From both, physicochemical (low molecular weight 384.3g/mol, low dose 5-20 mg) and pharmacokinetic (absolute bioavailability about 10-25 %) views, FDP is considered to be suitable for buccal delivery.

In the present study, matrix based bilayered buccoadhesive patches were developed and evaluated for *in vitro* drug permeation studies, buccal absorption, *in vitro* drug release, moisture absorption, mechanical properties, surface pH studies, *in vitro* bioadhesion and stability in human saliva.

Materials and Methods

Materials

Felodipine was gifted by Sun pharmaceuticals, Baroda, India. Hydroxypropyl methylcellulose E15 and Eudragit RLPO were gifted by Dr Reddys Laboratories Hyderabad, India. Polyester backing membrane was gifted by 3M, St. Paul, MI, USA. Mucin (Crude Type II) was procured from Sigma-Aldrich, Germany and was used without further purification. Phosphate buffer saline, Dulbecco's and Phenol red were purchased from Hi Media, Mumbai, India. All reagents used were of analytical grade.

Tissue preparation (Isolation)

Porcine buccal tissue from domestic pigs was obtained from local slaughterhouse and used within 2 hours of slaughter. The tissue was stored in Krebs buffer at 4° C after collection. The epithelium was separated from the underlying connective tissue with surgical technique and the delipidized membrane was allowed to equilibrate for approximately one hour in receptor buffer to regain the lost elasticity.

In vitro drug permeation studies

The buccal epithelium was carefully mounted between the two compartments of a Franz diffusion cell with an internal diameter of 2.1 cm (3.46 cm^2 area) with a receptor compartment volume of 25.0 mL. Phosphate buffer saline (PBS) pH 7.4 containing 40 % v/v of polyethylene glycol (PEG 400) and 10 % v/v alcohol was placed in the receptor compartment. The donor compartment contained 4 mL solution of PBS pH 7.4 and PEG 400 (1:1) in which 5 mg of FDP was dissolved. The donor compartment also contained phenol red a non absorbable marker compound at a concentration of 20 ìg mL^{-1}. The entire set up was placed over magnetic stirrer and temperature was maintained at 37° C. Samples of 1 mL were collected at predetermined time points from receptor compartment and replaced with an equal volume of fresh solution (12, 13).

Buccal absorption studies

Buccal absorption test was performed for FDP solution in 8 healthy male volunteers aged between 24 and 29 years and weighing between 60 to 75 kg. The ethics committee of the University College of Pharmaceutical Sciences, Kakatiya University, India, approved the protocol. This method uses phenol red, a non absorbable marker for determining saliva volumes. Phenol red is lost neither by absorption nor by swallowing (14, 15). Before the test, volunteers were asked to moisten their mouth with 20 mL of buffer solution. PBS pH 6.6 (20 mL) containing 4 mg FDP and phenol red (20 µg mL^{-1}) was given to volunteers and were asked to swirl the solution about 60 swirling min^{-1}. The samples of 1 mL were collected from the floor of the mouth at 2, 4, 6, 8, 10, 12, 14, and 16 min using a micropipette.

While collecting the samples, volunteers were asked to stop swirling momentarily. After the last sample was collected, all the solution was expelled into beaker. Volunteers were asked to rinse their mouth twice with 20 mL of PBS pH 6.6 and the washings were pooled with the original sample. Volume was noted and the quantity of FDP present in the samples was estimated by high performance liquid chromatography (HPLC). Phenol red was estimated colorimetrically by making the solution alkaline with sodium hydroxide.

Estimation of drug content by HPLC

Analysis of samples was performed using HPLC. The HPLC system (Shimadzu, Kyoto, Japan) consisting of a LC-10AT solvent module, SPD10A UV–visible detector with LC10 software. The analytical column used was C18 column (Inertsil, 150mm x 4.6mm i.d., particle size 5 µm) at an ambient temperature. The mobile phase used was a mixture of (66:34) of acetonitrile and water. The flow rate was 1 mL min^{-1} and detection was carried out at 240 nm. A calibration curve was plotted for FDP in the concentration range of 0.5-10 µg mL^{-1}. A good linear relationship was observed between the concentration of FDP and the peak area of FDP with a correlation coefficient ($r^2 = 0.999$). The required studies were carried out to estimate the precision and accuracy of the HPLC method.

Preparation of bilayered mucoadhesive buccal patches

Bilayered mucoadhesive buccal patches were prepared using solvent casting technique with HPMC E15 as primary polymeric layer, Eudragit RLPO as secondary polymeric layer and propylene glycol as plasticizer. Primary polymer was added to 25 mL of solvent mixture (dichloromethane and methanol, 1:1) and allowed to stand for 6 hr to swell. Propylene glycol and FDP were dissolved in 5 mL of solvent mixture and added to the polymeric solution. This was set aside for 2 hr to remove entrapped air, transferred to a Petri plate, and dried at room temperature. The secondary polymeric solution was prepared by dissolving 600 mg of Eudragit RLPO and 120 µL of propylene glycol in 15 mL of solvent mixture and poured on the primary polymer layer and allowed for drying at room temperature. The developed patches were removed carefully, cut to size (each having an area of 1.13 cm^2), and stored in a desiccator. The composition of the patches was shown in Table 1. Patches were subjected to weight variation, thickness variation and content uniformity. Patches with any imperfections, entrapped air, differences in weight were excluded from further studies.

Table 1. Composition of bilayered buccal patches of felodipine

Component	BB1	BB2	BB3	BB4	BB5	BB6
Primary layer						
Felodipine (mg)	320	320	320	320	320	320
HPMC E15 (mg)	1750	2000	2250	2500	2750	3000
Propylene glycol (µL)	262.5	300	337.5	375	412.5	450
Secondary layer						
Eudragit RLPO (mg)	600	600	600	600	600	600
Propylene glycol (µL)	120	120	120	120	120	120

In vitro release studies

The drug release from buccal patches was studied using USP type II dissolution test apparatus (Lab India dissolution test apparatus, Disso 2000, Mumbai, India) equipped with an auto sampler and fraction collector for the collection and replenishment of the sample and dissolution medium, respectively. Patches (1.13 cm^2) were meant to release drug from one side only; therefore, an adhesive impermeable polyester backing layer was placed on the other side of patch. The assembly for release studies was prepared by sandwiching the patch between dialysis membrane 50 KD (Hi Media, Mumbai, India). A piece of glass slide was placed as support to prevent the assembly from floating. The dialysis tubing with patch inside was secured from both ends using dialysis closure clips and placed in the dissolution apparatus. The dissolution medium was 500 mL of 0.5 % w/v of sodium lauryl sulphate solution at 25 rpm and temperature was maintained at 37° C. Samples of 5 mL were collected at predetermined time intervals and analyzed spectrophotometrically at 240 nm.

Moisture absorption studies

The moisture absorption studies give an indication about the relative moisture absorption capacities of polymers and an idea whether the formulations maintain their integrity after absorption of moisture. Moisture absorption studies were performed in accordance with the procedure reported earlier (16). Briefly, 5 % w/v agar in distilled water, which in hot condition was transferred to Petri plates and allowed to solidify. Then 6 patches from each formulation were weighed and placed over the surface of the agar and left for 2 hr at 37° C and the patch was weighed again. The percentage of moisture absorbed was calculated using the formula:

% Moisture absorbed = [(Final weight - Initial weight)/Initial weight] X100

Measurement of mechanical properties

Mechanical properties of the patches were evaluated using a microprocessor based advanced force gauze with a motorized test stand (Ultra Test, Mecmesin, West Sussex, UK) and fitted with a 25 kg load cell. Strips from the patch with dimensions of 60 x 10 mm and no visual defects were cut and positioned between two clamps separated by a distance of 3 cm. Clamps were designed to secure the patch without crushing it. During test, lower clamp was held stationary and the strips were pulled apart by the upper clamp moving at a rate of 2.0 mm/sec until the strip broke. The force and elongation of film at the point when the strip broke were recorded. The tensile strength and elongation at break values were calculated using the formula:

Tensile strength ($kg.mm^{-2}$) = [Force at break (kg)/Initial cross sectional area of the sample (mm^2)]

Elongation at break ($\%mm^{-2}$) = [Increase in length (mm) ×100]/ [Original length×Cross-sectional area (mm^2)]

Surface pH study

The method adopted by Bottenberg *et al.* (17) was used to determine the surface pH of the patches. A combined glass electrode was used for this purpose. The patches were allowed to swell by keeping them in contact with 1 mL of distilled water (pH 6.5 ± 0.1) for 2 h at room temperature, and pH was noted down by bringing the electrode in contact with the surface of the patch, allowing it to equilibrate for 1 minute.

In vitro bioadhesion measurement

The adhesive binding of the patches containing FDP to porcine buccal mucosa was studied in triplicate with the same equipment as the one used for measurement of mechanical properties except that a load cell of 5 kg was used for this study. In this test, porcine buccal

membrane was secured tightly to a circular stainless steel adaptor and the buccal patch to be tested was adhered to another cylindrical stainless steel adaptor similar in diameter using a cyanoacrylate adhesive. During test, 100 µL of 1% w/v mucin solution was spread over the surface of the buccal mucosa and the patch was immediately brought into contact. A force of 0.5 N was applied for 180 sec to enhance the contact of the patch with the mucosa. At the end of the contact time, upper support was withdrawn at a speed of 0.5 mm sec^{-1} until the patch was completely detached from the mucosa (18). The work of adhesion was determined from the area under force-distance curve while the peak detachment force was the maximum force required to detach the patch from the mucosa.

In vitro permeation of felodipine through porcine buccal membrane from bilayered buccal patch

In vitro permeation of FDP from bilayered buccal patches for the selected formulation (BB4) through porcine buccal membrane was studied. Buccal membrane was isolated as described in tissue preparation section. The membrane was mounted over a Franz diffusion cell whose internal diameter is 2.1 cm. The buccal patch was sandwiched between the buccal mucosa and the dialysis membrane, so as to secure the patch tightly from getting dislodged from the buccal membrane. The entire set up was placed over magnetic stirrer and temperature was maintained at 37° C. Samples of 1 mL were collected at predetermined time points from receptor compartment and replaced with an equal volume of fresh solution.

Stability in human saliva

The stability of optimized patches was performed in natural human saliva which was collected from humans aged between 18 to 35 years and filtered. Patches were placed in separate Petri plates containing 5 mL of human saliva and kept at a temperature controlled oven (Sheldon Manufacturing Inc., Cornelius, USA) at 37 ± 0.2° C for 6 h. At regular time intervals (0, 0.5, 1, 2, 4 and 6 h), the patches were examined for changes in color and shape, collapse of the patch and drug content. Drug content was determined by appropriate dilution of human saliva in phosphate buffer pH 6.8 and analyzed by spectrophotometry at 240 nm (19).

FTIR studies

The FDP, HPMC E 15 and physical mixture of FDP were prepared. The samples were prepared by grounding the pure drug, polymer and physical mixture with KBr separately. The IR spectra for the samples were obtained using KBr disk method using an FTIR spectrophotometer (PERKIN ELMER FT-I Insf. USA).

Results and Discussion

Drug penetration studies through porcine buccal membrane

Porcine buccal mucosa has been the most frequently chosen model for *in vitro* permeation studies because of its similarity to human tissue and is available in large quantities from slaughterhouses. Cumulative percentage amount permeated in 6 h was found to be 65.2 ± 2.48 % and the flux was calculated to be 0.153 mg $h^{-1}cm^{-2}$. The penetration of drug through the porcine buccal epithelium was found to be rapid up to first 3 hours followed by a slow penetration in the next 3 hours (Fig.1). The tissue could be isolated successfully because no detectable levels of phenol red (marker compound) was found in the receiver compartment, where as FDP could penetrate freely.

Buccal absorption study

Buccal absorption study was conducted to substantiate the results from the *in vitro* permeation studies. In addition, it gives

information regarding the irritant nature of the drug to oral mucosa. The results of buccal absorption study are shown in Fig. 2. It was observed that about 56.24 % of the drug was absorbed through the buccal membrane in 16 min. The drug was absorbed at a rapid rate for the first 2 min, after which the drug absorption continued at a uniform rate. The total amount of phenol red present in 8 collected samples was found to be the same when compared to the initial collected samples of phenol red (400 µg) in solution. This indicated that the volunteers did not swallow the solution. The total amount of saliva secreted during 16 min of study was found to be averaging 26.42 mL. The volunteers reported numbness in the mouth for about 15 to 20 min after the test. The results of buccal absorption study revealed that FDP could penetrate through the oral cavity.

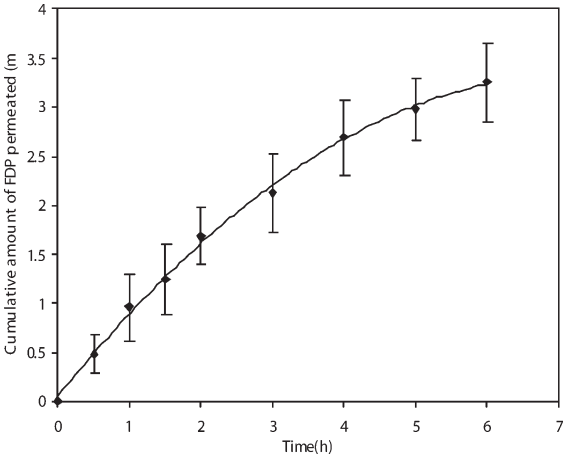


Fig. 1 *In vitro* permeation of FDP through porcine buccal mucosa, the values represented mean ± S.D (n=3)

Mass, thickness and drug content determination

Preliminary trials were conducted to develop FDP buccoadhesive patches using HPMC E15 as polymer. The results reveal that

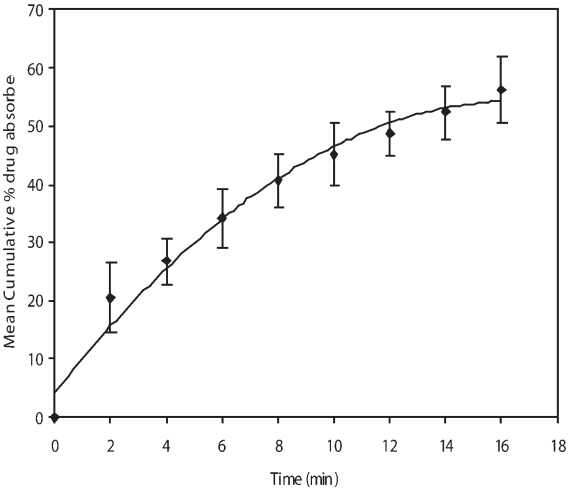


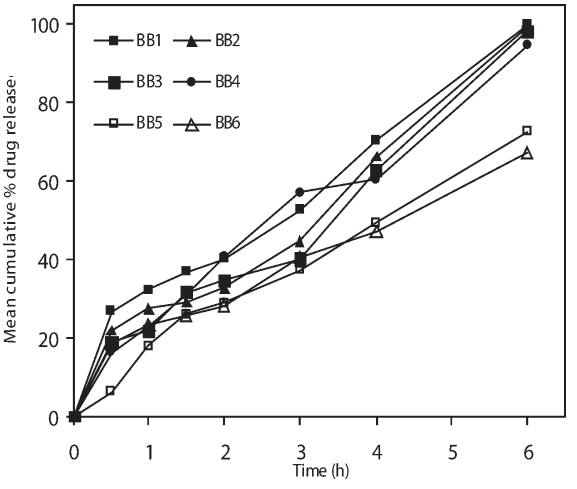
Fig.2 Buccal absorption of FDP in healthy human volunteers, the values represented mean ± S.D (n=8)

the drug was diffused from the patches on to the surface. Therefore to overcome the problem, bilayered patches were developed using Eudragit RLPO as secondary layer. The drug diffusion from patches was prevented by the secondary layer composed of Eudragit RLPO. The prepared patches were smooth in appearance, uniform in thickness, mass, and drug content and showed no visible cracks. The mass of patches ranged from 62 ± 2 to 72 ± 2 mg and the thickness ranged from 510.0 ± 7.1 to 580.2 ± 4.3 µm. The drug content in the buccal patches ranged from 96.7 ± 0.5 to 99.6 ± 0.2 %, indicating the favorable drug loading and patches uniformity with respect to drug content.

In vitro drug release studies

The drug release profiles of FDP from buccal patches are shown in Fig. 3. It was clear from the plots, the drug release was governed by polymer content. No lag time was observed as the patch was directly exposed to the dissolution medium. An increase in the polymer content was associated with decrease in drug release rates. The description of drug release profiles by a model

function has been attempted using zero order and first order; release pattern using Korsmeyer et al (20)



$M_t/Má=K.t^n$

Where Mt/Má is the fractional release of drug, Mt is the amount released at time t, Má is the total amount of drug contained in the patches, t is the release time, K is the kinetic constant and n is the release exponent indicative of the operating release mechanism.

Formulation BB1 showed maximum drug release among the formulations. The drug release ranged from 67.1 (BB6) to 99.8 % (BB1). However the difference among the formulations (BB1, BB2, BB3 and BB4) was statistically insignificant. Formulations BB1, BB2 and BB3 (r^2>0.98) followed first order release kinetics, where as BB4, BB5 and BB6 (r^2>0.99) showed zero order release kinetics as it was evidenced from correlation coefficients. All formulations showed non-fickian of release pattern as it was evidenced from release exponent (n>0.51) (21). Increasing the amount of the polymer in the patches produced the water-swollen gel like state that could substantially reduce the penetration of the dissolution medium into the patches and so the drug release was delayed. The Eudragit layer minimizes the diffusion of the drug molecules from the patches. In addition, Eudragit layer could control the release of the drug from the patches. This was evidenced from the release studies of the monolayer patches where the drug release was rapid. Therefore a rate controlling membrane could be used to control the release. The formulation that showed maximum amount of drug release with zero order release kinetics was selected as the optimized formulation and further was used for the evaluation of *in vitro* permeation studies across porcine buccal membrane and *in vitro* bioadhesion studies.

Table 2. Physical and mechanical parameters of bilayered buccal patches of felodipine

Batch code	Mass[a] (mg)	Thickness[a] (µm)	Drug Content[a] (%)	Surface pH[a]	Mean% Moisture Absorbed[a]	Tensile Strength[a] (Kg/mm^2)	Elongation at Break[a] (% mm^2)
BB1	62 ± 2	510 ± 70	97.3 ± 0.5	5.9 ± 0.2	68.4 ± 2.2	2.84 ± 0.1	17.7 ± 3.2
BB2	64 ± 2	530 ± 50	98.9 ± 1.1	5.8 ± 0.1	77.9 ± 2.3	4.28 ± 1.2	18.9 ± 2.9
BB3	65 ± 1	550 ± 90	97.3 ± 0.4	6.0 ± 0.1	64.2 ± 1.8	7.49 ± 2.4	20.8 ± 3.8
BB4	68 ± 1	560 ± 40	99.6 ± 0.2	5.9 ± 0.1	74.6 ± 2.6	8.45 ± 2.2	22.2 ± 4.2
BB5	70 ± 2	570 ± 20	96.6 ± 0.5	5.8 ± 0.1	60.4 ± 3.4	10.4 ± 2.1	38.6 ± 5.2
BB6	72 ± 2	580 ± 40	96.9 ± 0.6	6.0 ± 0.1	66.7 ± 2.8	12.1 ± 2.5	56.4 ± 5.5

[a] Mean ± SD, $n = 3$

Moisture absorption studies

Moisture absorption studies evaluated the integrity of the formulation upon exposure to moisture. The results of moisture absorption studies, mass, thickness, drug content and surface pH were presented in Table 2. Results showed that there are differences in moisture absorption with BB1 to BB6 the percentage moisture absorbed ranged from about 60.4 to 77.9 % w/w for various formulations. When the patches were placed without backing membrane complete swelling followed by erosion was observed indicating that the drug release mechanism involves swelling of the polymer initially followed by drug release from the swollen matrix by diffusion.

Mechanical properties of films

Ideal buccal film, apart from good bioadhesive strength, should be flexible, elastic, and strong enough to withstand breakage due to stress caused during its residence in the mouth. The tensile strength (TS) and elongation at break (E/B) shows the strength and elasticity of the film. A soft and weak polymer is characterized by a low TS and E/B; a hard and brittle polymer is defined by a moderate TS, and low E/B; a soft and tough polymer is characterized by a moderate TS and a high E/B; whereas a hard and tough polymer is characterized by high TS and E/B (22). An ideal buccal film should have a relatively high TS and E/B (9). The results of the mechanical properties, i.e., TS and E/B, are presented in Table 2. TS and E/B increased with the increase in polymer content. Maximum TS was exhibited by BB6 (12.1 ± 2.5 kg.mm^{-2}) which was statistically significant different ($p<0.05$) compared to BB1 (2.8 ± 0.1 kg.mm^{-2}). Formulation BB4 showed 8.45 Kg. mm^{-2} and 22.2 % mm^2 of TS and E/B respectively. Maximum E/B was seen with BB6 (56.4 ± 5.5 % mm^{-2}) and the least was observed with BB1 (17.7 ± 3.2 % mm^{-2}).

Surface pH studies

The surface pH of the patches was determined in order to investigate the possibility of any side effects, *in vivo*. Since an acidic or alkaline pH may cause irritation to the buccal mucosa, we attempted to keep the surface pH as close to neutral as possible. The surface pH of all the patches (BB1 to BB6) was near 6 and hence, these patches should not cause any irritation in the buccal cavity.

In vitro bioadhesion studies

In vitro bioadhesion measurements are performed routinely for mucoadhesive dosage forms, and the most commonly used technique for evaluation of buccal patches is the measurement of adhesive strength (23).Work of adhesion, calculated from area under the force distance-curve, is a measure of work that must be done to remove a patch or film from the tissue. Peak detachment force is the maximum applied force at which the patch detaches from tissue. The peak detachment force and work of adhesion for the BB4 patch was calculated as 3.42 ± 0.54 N and 1.63 ± 0.16 mJ respectively. The work of adhesion and peak detachment force values was within the range for suitable bioadhesion as reported for various films (9). In addition, all the formulations were found to have similar values since the basic surface environment of the patch, which is essential for the bioadhesion, remains the same and it is only the thickness that varies. However, differences do exist due to change in the polymer type or composition of the film.

In vitro permeation of FDP through porcine buccal membrane from bilayered buccal patch

Formulation BB4 was selected for the *in vitro* permeation studies due to its superior drug release properties in terms of percentage drug released, its capacity to retain the structure in

moisture absorption studies, and bioadhesion studies *in vitro*. The results (Fig. 4) indicated that the drug permeation was slow and about 41.6 % of FDP could permeate through the buccal membrane with a flux of 0.113 mg $h^{-1}cm^{-2}$ in 6 hours. The results of drug permeation reveal that FDP was released from the formulation and permeated through porcine buccal membrane and hence could possibly permeate through the human buccal membrane.

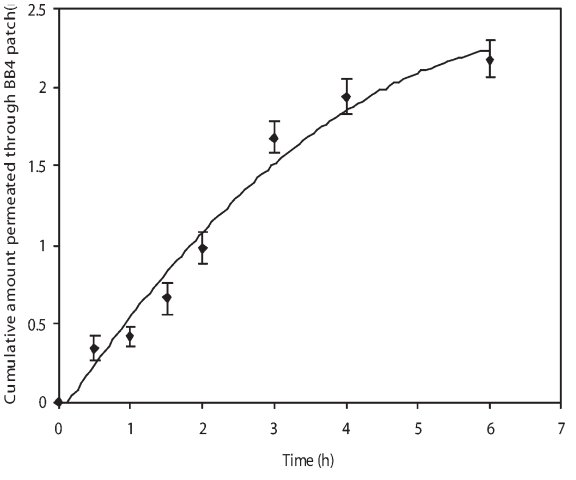


Fig. 4 *In vitro* permeation of FDP from patch BB4 through porcine buccal mucosa, the values represented mean ± S.D (n=3)

Stability of patches in human saliva

Stability studies are usually performed in phosphate buffer solution whose pH pertains to the buccal cavity, but stability studies performed in normal human saliva would be more appropriate to mimic the stability of drug and device in the oral cavity *in vivo*. Therefore, the stability study of optimized patches (BB4) was examined in human saliva and their appearance characteristics, such as color, shape and drug content in natural human saliva were evaluated (Table 3). Thickness and diameter of patches increased to 20.2 and 5.4 % owing to swelling in human saliva in 6 h studies. No color changes were observed. The recovery of the drug from all patches was found to be 97.9 % indicating maximum utilization of the drug incorporated.

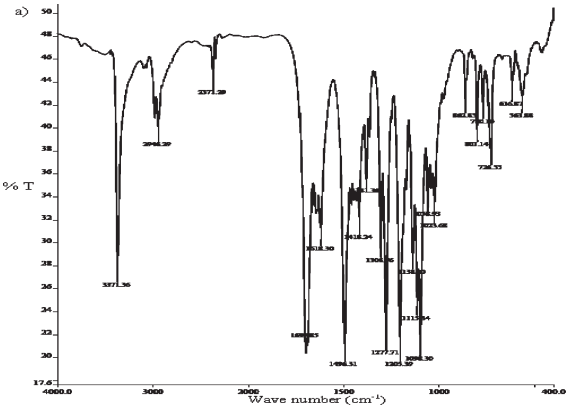
FTIR Studies

To study any interaction between drug and polymers used in the preparation of patches IR spectroscopic studies were carried out. Figs 5a–c shows IR spectra of the FDP, HPMC E15 and physical mixture (BB4). FDP alone showed principal peaks at 1699.85, 1496.31, 1205.39, and 1098.30 cm^{-1}. HPMC E15 was not showed any characteristic peaks except few broad peaks at 1062.2, 2931.4 and 3471.5 cm^{-1}. The IR spectra of the physical mixture showed the same absorption bands as the pure drug, illustrating absence of interaction between FDP and HPMC E15.

Table 3. Stability study of optimized bilayered buccal patch (BB4) in human saliva

Sampling time (h)	Thickness (mm)[a]	Diameter (mm)[a]	Drug recovered (%)[a]
0	0.46 ± 0.05	12.0 ± 0.1	99.6 ± 0.2
0.5	0.48 ± 0.06	12.1 ± 0.1	97.8 ± 0.4
1	0.53 ± 0.08	12.2 ± 0.1	98.2 ± 0.6
2	0.56 ± 0.05	12.2 ± 0.2	98.4 ± 0.5
4	0.58 ± 0.06	12.4 ± 0.2	98.8 ± 0.3
6	0.60 ± 0.03	12.5 ± 0.3	99.3 ± 0.2

[a] Mean ± SD, $n = 3$.



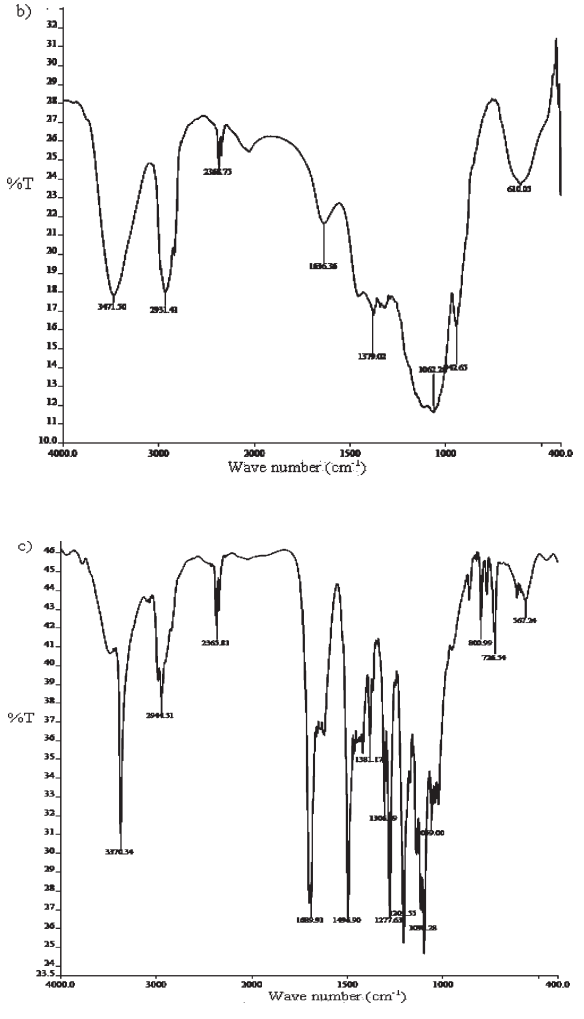


Fig.5. FTIR spectra of: a) FDP, b) HPMC E15 and c) FDP bilayered buccal patch (BB4)

Conclusions

Bilayered buccoadhesive patches for buccal delivery of felodipine could be prepared. Formulation BB4 using the mucoadhesive polymer at a ratio 1:8 showed significant bioadhesive properties with an optimum release profile and could be useful for buccal delivery. Further work is recommended to support its efficacy claims by long term pharmacokinetic and pharmacodynamic studies in human beings.

Acknowledgements

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Simple, Rapid and Sensitive Method for Determination of Tacrolimus in Human Blood by using Liquid Chromatography / Tandem Mass Spectrometry

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Abstract

A simple, rapid and sensitive method using an isocratic Liquid chromatography coupled with Tandem mass spectrometry was developed and validated for the assay of Tacrolimus in the Human Blood. The Mass transition of Tacrolimus and Sirolimus (Internal standard) were M/z 821.7/768.7 and M/Z 931.6/864.6 in ESI Negative ionization. Linearity was observed between the Tacrolimus concentration and the peak area ratio from 0.52 to 61.00 ng/mL with r2 value of 0.99. Blood samples containing Tacrolimus were extracted with Zinc sulphate and methanol to precipitate proteins followed by SPE. The observed recovery of Tacrolimus was 66.7 %. The intra-day and inter-day accuracy ranged from 91.3 – 110.5% and from 93.9 – 100.6% respectively, at Low, middle and high level concentrations. The method will be used in the determination of the pharmacokinetic parameters of Tacrolimus after oral administration of Tacrolimus formulation in human blood.

Keywords: Tacrolimus, LCMS/MS, Human Blood

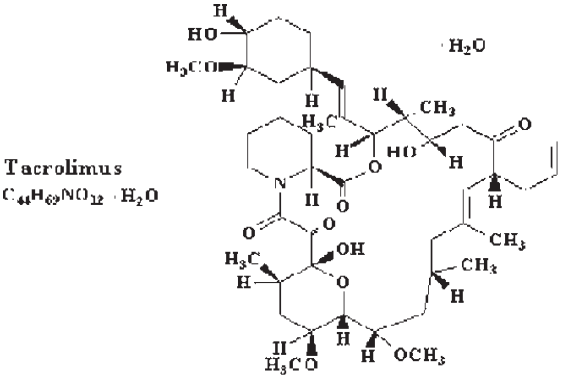
Introduction

Tacrolimus is a macrolide immunosuppressant (1) and has unpredictable pharmacokinetics, therefore regular monitoring is required in patients receiving Tacrolimus. It reduces peptidyl-prolyl isomerase activity by binding to the immunophilin FKBP12 (FK506 binding protein) creating a new complex. This FKBP12-FK506 complex interacts with and inhibits calcineurin thus inhibiting both T-lymphocyte signal transduction and IL-2 transcription (2, 3, 4). It also prevents the dephosphorylation of NF-AT (5). It is used in organ transplantation to prevent graft rejection. Even if the Tacrolimus activity is similar to cyclosporin, studies have shown that the incidence of acute rejection is reduced by tacrolimus use over cyclosporin (6).

Although short-term immunosuppression concerning patient and graft survival is found to be similar between the two drugs, tacrolimus results in a more favorable lipid profile, and this may have important long-term implications given the prognostic influence of rejection on graft survival (7).However, its use is not devoid of side effects, making it important to maintain blood concentrations within therapeutic ranges (8,9-16). Several analytical methods are currently available for routine drug monitoring (17,18-22,23).We have developed a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method (24,25-28,29) for the quantification of Tacrolimus concentrations in whole blood.

Stricture of Tacrolimus:

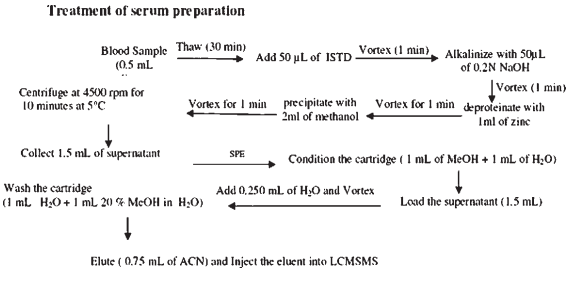
Fig.1



Materials and Methods

Materials: Tacrolimus and Sirolimus analytical standards were obtained from Biocon India Pvt Ltd, HPLC grade Acetonitrile and methanol were obtained J.T. Baker, USA, GR grade Ammonium acetate, Sodium hydroxide and Zinc sulphate were obtained from Merck, USA, HLB (Hydrophilic-lipophilic balance) Solid Phase Extraction cartridges were purchased from Water's corporation, Milford, MA USA Deionized and triple distilled water was used for the process.

Preparation of Tacrolimus standard solutions: Standard solution of Tacrolimus (1.0 mg/ml) was prepared by dissolving an accurately weighed amount of 10.27 mg of Tacrolimus (99.4% purity on as is basis) in 5 ml of methanol, sonicated for 5 minutes and the volume was adjusted to 10 ml with methanol. The standard solution was stored subsequently at 2-8 0C. The appropriate concentrations of standard solution were prepared by diluting the stock solution with 80: 20 methanol: water.



Preparation of Sirolimus Internal standard solutions: Standard solution of Sirolimus (1.0 mg/ml) was prepared by dissolving an accurately weighed amount of 10.22 mg of Sirolimus in 5 ml of methanol, sonicated for 5 minutes and the volume was adjusted to 10 ml with methanol. The standard solution was stored subsequently at 2-8 0C. 2.5µg/mL Sirolilmus was prepared by diluting the stock solution with 80: 20 methanol : water.

***Instrumentation and conditions*:** The Agilent HPLC comprises Pump (Model: 1100 series), auto-sampler (Model: 1200 series) and auto injector coupled with Triple quadrupole AB Sciex API-3000 LC-MS/MS system interfaced to analyst software in a windows platform. The Reverse phase C18 analytical column (Thermo Hypersil Hypurity, Length: 4.6×50 mm, Particle size: 5 µ) was used for the separation of Tacrolimus and internal standard, the mobile phase consists a mixture of methanol and 5mM Ammonium Acetate at a ratio of 90:10 respectively. The mobile phase was degassed by passing through a 0.22 µm membrane filter (Millipore, Bedford, MA, USA) prior to use. The mobile phase was pumped isocratically at a flow rate of 0.5 ml/min. The injector was filled with an injector loop of 10 µl. Tandem mass spectrometric detection and quantification was performed using multiple reaction monitoring (MRM).

Results and Discussion

Development conditions for rapid extraction of Tacrolimus from Human Blood: The extraction procedure developed for Tacrolimus from Human Blood allowed samples to be available for HPLC-UV analysis in approximately 30 minutes.(!9) Conditions for simple and rapid HPLC separation with MS/MS Electro-spray Negative

ionization mode were developed using an isocratic elution with a mobile phase composed of methanol and 5mM Ammonium acetate at a ratio of 90:10% v/v. Thus the ions formed for drug and Internal standard in ESI Negative mode due to the addition of Hydroxyl ion (OH^-) in carbonyl function (C=O) present in the drug (Fig.1). These conditions gave a well defined, sharp peak of Tacrolimus and Sirolimus (ISTD) with a retention time of approximately 1.78 minutes for both. Under these conditions an amount of Tacrolimus as low as 10pg/mL could be detected. With these retention times, analysis could be completed in about 2.5 minutes. Sirolimus also falls under the same category of immunosuppressant. More over using the same nature of molecule (Sirolimus)has improved the efficiency of the method.

Method validation

Linearity: The quantification of the chromatogram was performed using the peak area ratio of Tacrolimus and Sirolimus (ISTD). Nine standard solutions were prepared (0.52 1.03, 2.20, 6.29, 12.27, 12.27, 25.14, 36.97, 49.30 ng/mL and 61.62 ng/mL) and subjected analyses by HPLC-MS/MS. Five precision and accuracy (P&A) batches with ten calibration curve standards were injected. The peak area ratio was determined and plotted versus the concentration of Tacrolimus. Statistical analysis using least square regression analysis indicated excellent linearity for Tacrolimus with the concentration range studied as shown in Table 1. In constructing the standard curve, samples of Tacrolimus in Human Blood identical to those in the standard solutions were prepared and the Tacrolimus response ratios were plotted against the concentrations of Tacrolimus in ng/mL as shown in Fig. 2. The linearity of the concentration and response relation was established over the range of 0.52 – 61.62 ng/mL (R2 = 0.9898). Fig. 3 shows the LC-MS/MS chromato-grams of pure drug. (Tacrolimus), Fig. 4 shows the LC-MS/MS chromatograms of drug-free Human Blood and Fig. 5 shows the LC-MS/MS chromatograms of standard Blood sample containing the drug at a concentration of 0.52 ng/mL.

Accuracy and precision: The intra-day accuracy and precision of the assay was evaluated by analyzing six replicates of the Blood containing

Table 1: Curve parameter summary and back-calculated calibration curve concentration for Tacrolimus in whole human blood

	Back calculated concentrations for calibration curve standards													
	STD 9-1	STD 9-2	STD 8	STD 7	STD 6	STD 5	STD 4	STD 3	STD 2	STD 1-1	STD 1-2			
CC ID	Nominal Value (ng/mL)													
	61.62	61.62	49.30	36.97	25.14	12.27	6.29	2.20	1.03	0.52	0.52	Slope	Intercept	r2
Maximum	70.86	70.86	56.70	42.52	28.91	14.11	7.23	2.53	1.18	0.62	0.62			
Minimum	52.38	52.38	41.91	31.42	21.37	10.43	5.35	1.87	0.88	0.42	0.42			
CC1	62.69a	59.75	52.17	36.94	25.33	12.42	6.43	1.92	1.14	0.50a	0.51	0.0389	-0.000847	0.9946
CC2	64.54a	58.96	49.89	38.35	24.8	12.77	6.37	1.94	1.18	0.5	0.47a	0.038	-0.00107	0.9834
CC3	65.45a	59.29	49.31	37.18	25.7	11.99	6.52	1.80a	1.07	0.49a	0.51	0.0407	0.0000575	0.9986
CC4	61.67	64.09a	51.89	37.98	26.49	13.44	5.78	2.03	0.9	0.57a	0.56	0.0406	-0.00292	0.9924
CC5	60.34	65.68a	52.50	39.56	26.01	13.73	5.62	1.99	0.90	0.57	0.61a	0.0392	-0.00178	0.9898
Mean	61.005	59.125	50.898	38.268	25.750	12.983	6.073	1.987	1.013	0.535	0.535			
SD ±	0.9405	0.2333	1.5371	0.9904	0.7119	0.7742	0.4393	0.0451	0.1374	0.0495	0.0354			
%CV	1.5	0.4	3.0	2.6	2.8	6.0	7.2	2.3	13.6	9.3	6.6			
%Nominal	99.0	96.0	103.2	103.5	102.4	105.8	96.5	90.3	98.3	102.9	102.9			

Acceptance Criteria: 1) Precision for all CC std 1-8 should be within 15% and for LOQ should be within 20%.
2) Accuracy for all CC std 1-8 should be within 85-115% and for LOQ should be within 80-120%.

Note:- a - Standard was not considered for calculation.
NA- Not Applicable

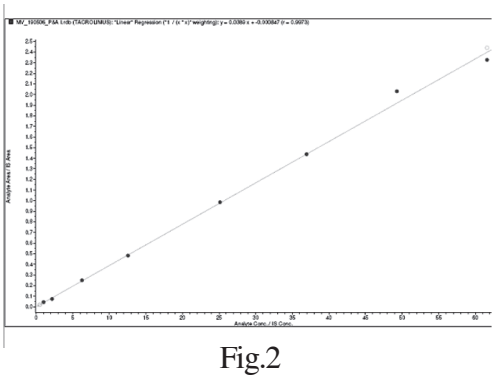


Fig.2

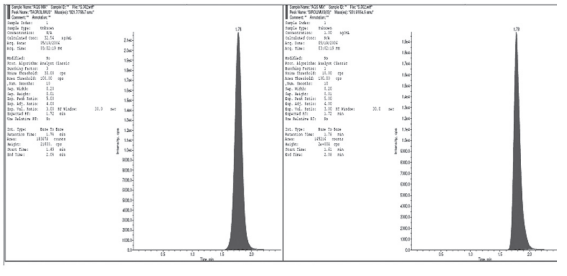


Fig.3

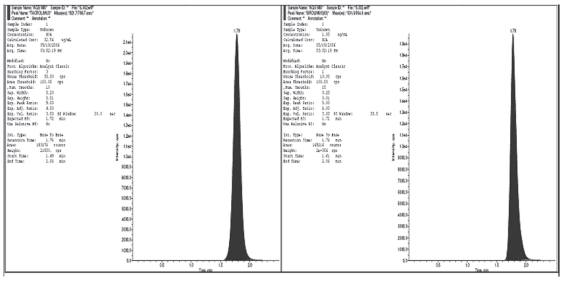


Fig.4

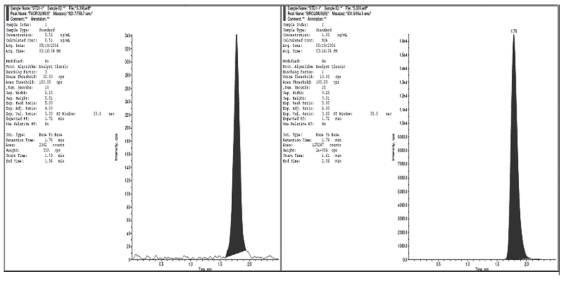


Fig.5

Table 2: Within and between batch precision and accuracy

	QC No.	HQC	MQC	LQC	LOQ QC
		Actual concentration (ng/mL)			
		55.46	27.73	1.66	0.53
Maximum limit		63.78	31.89	1.91	0.64
Minimum limit		47.14	23.57	1.41	0.42
P&A I	1	54.59	26.27	1.58	0.54
	2	52.11	25.55	1.54	0.53
	3	52.25	24.89	1.56	0.63
	4	52.00	25.52	1.52	0.52
	5	52.29	24.75	1.57	0.57
	6	49.32	24.86	1.62	0.52
	Mean	52.093	25.307	1.565	0.552
	S.D +	1.6731	0.5858	0.0345	0.0426
	% CV	3.2	2.3	2.2	7.7
	% Nominal	93.9	91.3	94.3	104.1
P&A II	7	51.59	26.73	1.62	0.53
	8	53.39	24.87	1.61	0.56
	9	51.66	25.25	1.67	0.57
	10	53.13	25.25	1.68	0.55
	11	49.87	25.59	1.51	0.53
	12	50.65	24.83	1.67	0.61
	Mean	51.715	25.420	1.627	0.558
	S.D +	1.3686	0.7005	0.0641	0.0299
	% CV	2.6	2.8	3.9	5.4
	% Nominal	93.2	91.7	98.0	105.3
P&A III	13	56.48	26.25	1.48	0.49
	14	52.77	24.42	1.61	0.52
	15	52.74	24.67	1.59	0.49
	16	56.21	26.45	1.48	0.45
	17	54.48	24.33	1.56	0.47
	18	52.74	25.85	1.56	0.56
	Mean	54.237	25.328	1.547	0.497
	S.D +	1.7673	0.9628	0.0550	0.0388
	% CV	3.3	3.8	3.6	7.8
	% Nominal	97.8	91.3	93.2	93.7
P&A IV	19	50.39	26.72	1.76	0.59
	20	58.29	26.77	1.84	0.61
	21	52.06	26.07	1.87	0.61
	22	53.27	26.68	1.77	0.59
	23	55.81	25.68	1.69	0.60
	24	52.69	27.26	1.73	0.57
	Mean	53.752	26.530	1.777	0.595
	S.D +	2.841	0.563	0.067	0.015
	% CV	5.3	2.1	3.8	2.5
	% Nominal	96.9	95.7	107.0	112.3
P&A IV	19	54.67	28.28	1.87	0.68
	20	55.39	27.00	1.93	0.59
	21	55.03	27.73	1.79	0.59
	22	53.16	27.20	1.82	0.61
	23	57.30	27.00	1.87	0.61
	24	51.97	28.61	1.73	0.61
	Mean	54.587	27.637	1.835	0.615
	S.D +	1.849	0.689	0.070	0.033
	% CV	3.4	2.5	3.8	5.4
	% Nominal	98.4	99.7	110.5	116.0
P&A V Ruggedness	25	54.67	28.28	1.87	0.68
	26	55.39	27	1.93	0.59
	27	55.03	27.73	1.79	0.59
	28	53.16	27.2	1.82	0.61
	29	57.3	27	1.87	0.61
	30	51.97	28.61	1.73	0.61
	Mean	54.587	27.637	1.835	0.615
	S.D +	1.849	0.689	0.070	0.033
	% CV	3.4	2.5	3.8	5.4
	% Nominal	1921.4	4911.4	2721.4	4055.2
Global statistics	Mean	53.277	26.044	1.670	0.563
	S.D +	2.171	1.146	0.130	0.052
	% CV	4.1	4.4	7.8	9.2
	% Nominal	96.1	93.9	100.6	106.3

Tacrolimus at three different concentrations. The intra-day precision of the analyzed samples as determined by R.S.D. (%) range from 4.1 to 9.2%, while the intra-day accuracy ranged from 93.9 – 100.6%. The inter-day precision of the assay was measured by analyzing six replicates of Tacrolimus Blood samples for three consecutive days. The inter-day precision of the analyzed samples as determined by R.S.D. (%) range from 2.1 to 7.8%, while the inter-day accuracy ranged from 91.3 – 110.5%.

Recovery: The absolute recovery was calculated by comparing the peak areas of Tacrolimus and Sirolimus standards to those assessed by extraction of Tacrolimus and Sirolimus at the three different concentrations. Results of absolute recovery of Tacrolimus and Sirolimus ranged from 60.00 to 83.42% and 60.66 to 82.25 respectively as shown in Table 2.

Conclusion

This method was validated in human blood for its specificity, sensitivity, linearity, accuracy, precision (repeatability & reproducibility), % recovery, stability of samples (Freeze thaw, Bench top and Auto sampler stability, short-term and long term stability of stock solution and Internal Standard), dilution integrity and for ruggedness. Method is applicable to quantify the Tacrolimus in clinical samples for further evaluation of pharmacokinetics.

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A Comparative Study on the Association of Labour process with Oxidative stress in Normal and Preeclamptic mothers

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Abstract

Oxidative stress plays an important role in the pathophysiology of preeclampsia. An increase in lipid peroxidation and decrease in antioxidant activity in preeclamptic women with hypertension have been reported. The present contribution aimed to understand the effect of labour on the levels of free radicals in normotesive pregnant mothers (control group) and hypertensive preeclamptic mothers. Serum MDA (Malondialdehyde) is used as a marker of oxidative stress mediated changes in the study subjects and the levels of enzymatic antioxidants viz SOD, GRx, GPx in the arterial blood (before delivery) and in cord blood are estimated. The results indicate that in hypertensive preeclamptic mothers, there is a significant ($p<0.001$) increase in the levels of serum MDA and a significant ($p<0.001$) decrease in the levels of antioxidant enzymes in cord blood when compared to control groups. This is attributed to the oxidative stress during labour pain. Hence it is inferred that antioxidant supplementation during pregnancy could prevent oxidative stress mediated changes to cell and biomolecules during the gestational period.

Key words: Oxidative stress, hypertension, pregnancy, lipid peroxidaton, antioxidant enzymes

Introduction

Preeclampsia is a specific disorder of pregnancy, characterized by hypertension, proteinuria and oedema developing in the second half and, leading to maternal and neonatal morbidity as well as mortality. It is characterized by endothelial cell dysfunction, lipid peroxidation, decreased antioxidants and alteration in immune responses (1-3). The endothelial changes are more appropriately described as dysfunction or activation of an altered state of endothelial cell differentiation in response to sublethal injury or cytokine stimulation (4), rather than damage to endothelium (5). The pathologic changes in the endothelial cells that line the renal glomerular capillaries (glomerular endotheliosis) are a consistent feature in the histopathology of women with pre-eclampsia (6, 7). Much evidence points to the role of Reactive Oxygen Species (ROS) in the etiology of hypertension and oxidative stress in preeclamptic mothers. ROS usually accompany increased peroxidation of lipids and is evidenced in the blood samples in the form of MDA. Free oxygen free radicals are highly reactive and are potentially damaging to most macromolecules like polyunsaturated fatty acids, proteins, carbohydrates and nucleic acids. Peroxidation of polyunsaturated fatty acids produces malondialdehyde. The presence of this oxidation product can be measured with thiobarbituric acid and its levels correlate with the extent of lipid peroxidation (8-11). Elevated level of lipid peroxide by-products like malondialdehyde is an indicator of oxidative

stress mediated changes in vivo (12). In the present study, we have investigated pair matched biochemical profile of oxidative stress indices and the levels of antioxidant defense enzymes. We evaluated in a comparative study of these enzymes in blood samples from maternal and cord blood of preeclamptic mothers and compared these data with those of normotensive mothers. We also studied the levels of these enzymes before and during the labour process, so as to estimate the role of labour as a stress factor.

The association between pregnancy-related hypertension and severity of hypertension (stage 2 according to Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure (JNC VII)) and end-organ damage was assessed in a logistic regression model. Gestational hypertension contribute to obstetrical complications and maternal mortality making it as a important health issue globally with a systolic blood pressure =140 mmHg and/or a diastolic blood pressure =90 mmHg, in the absence of proteinuria, in a previously normotensive pregnant woman at or after 20 weeks of gestation (13).

The objectives of this study were to investigate the levels of reactive oxygen species and those of antioxidant enzymes in maternal and cord blood samples of normotensive and preeclamptic mothers and their fetuses. The presence of pregnancy related hypertension is established by questionnaire and clinical examination.

Materials and Methods

Collection of Samples

Studies were conducted on blood samples from 40 patients falling in the age group of 25 to 35 years, who attended the Out Patient Department of Meenakshi Medical College and Research Institute, Chennai-600 078, Tamil Nadu. The study is cleared by Ethical Committee.

Experimental Setup

The patients were divided in to four groups of twenty patients each. Group I consisted of maternal blood samples of normotensive patients and served as a control. Cord blood samples from fetuses of normotensive mothers served as Group-II. Maternal blood samples of preeclamptic mothers formed Group III and cord blood samples of fetuses of preeclamptic mothers served as Group IV. Smoker, alcohol consumers and other drug abusers were excluded from the study. Demographic data pertaining to clinical characteristics of control and experimental subjects were documented. Blood Pressure at the time of delivery, pulse rate was documented using standardized procedures. Urine samples were analysed for protein. Clinical examination for pedal edema is documented in case report forms. Informed consent of the participants and clearance of the ethical committee was obtained prior to the onset of study. Maternal blood samples were collected at the time of delivery and cord blood samples from umbilical cord were collected immediately after delivery from the study participants (control and preeclamptic mothers). The samples were processed for the separation of plasma, red blood cells and haemolysate. Lipid peroxidation assay was estimated using plasma samples and haemolysate was used for the enzymatic antioxidants and the results were documented.

Lipid peroxidation

Lipid peroxidation in the plasma was estimated using TBA (Thiobarbituric acid) reaction method (14). Standard MDA 50mM solution of malondialdehyde was prepared in distilled water using 1,1,3,3 tetramethoxypropane. This was stored in 4OC and diluted just before use such that working standard contains 50 nM/ml. Plasma TBARS values were expressed in moles of MDA/L (15).

Assay of antioxidant enzymes

Preparation of hemolysate was done (16, 17). Blood collected with EDTA was centrifuged at 2000×g for 20 minutes at 4OC. The packed cells were washed with saline to remove the buffy coat. An aliquot of packed cells was washed with isotonic Tris – HCl buffer. 1.0 ml of washed cells was lysed using 9.0ml of hypotonic Tris – HCl buffer pH 7.2. The lysed cells were centrifuged at 15000 ×g for 30 minutes. The supernatant fraction (hemolysate) was used for the assay of antioxidant enzymes. The enzyme superoxide dismutase (SOD) was measured by the method of Hartz et al (18). The enzyme glutathione peroxidase (GPx) catalyses the reaction of ROS – like H2O2 leading to elevated LPO. GPx activitiy was measured spectrophotometrically following the method of Paglia and Valentine (19). Activity of glutathione reductase (GRx) was measured as described by Goldberg and Spooner (20).

Statistical analysis

Values are expressed as mean ± SD. Mean and Standard Deviation (SD) were estimated for different variables in each group. Mean values were compared between different study groups by using One way ANOVA. Values of $p<0.05$ were considered to be statistically significant.

Results

Table 1 depicts the demography, physiological status and clinical characteristics of normotensive and preeclamptic study participants. The mean maternal age was 29.1 ±6.9 in normotensive group while it was 26.8 ± 7.2 in preeclamptic subjects which is significant at ($p<0.275$) .Gestational age was 33.8 ±3.9 weeks in normotensive group while it was 32.2 ± 4.8 weeks in preeclamptic study participants which is significant ($p<0.221$). The systolic Blood Pressure at delivery is 109.8 ± 12.9 mm Hg in normotensive group and it was 164.5 ± 13.6 mm Hg in preeclamptic group which is significant ($p<0.0001$). The diastolic Blood pressure at delivery is 65.6 ± 11.4 mm Hg in normotensive group and it was 111.4 ± 14.2 mm Hg in preeclamptic group which is also significant ($p<0.0001$). Ischemia, reperfusion injury is known to be a potent source of free radicals like superoxide anion (O2-), Hydroxyl radicals (OH), Hydrogen peroxide H2O2 which again cause oxidative damage to cell membrane and vascular endothelium setting up a viscious cycle.

Table 1. Demographic and clinical characteristics of normotensive (Control) and severe preeclamptic subjects

Parameters	Normotensive control group Group I	Preeclamptic group Group III	P*-value
Number of maternal/neonatal pairs	20	20	
Maternal age (years)	29.1 ± 6.9	26.8 ± 7.2	0.275
Gestational age (weeks)	33.8 ± 3.9	32.2 ± 4.8	0.221
BP at delivery systolic (mm/Hg)	109.8 ± 12.9	164.5 ± 13.6	< 0.0001

BP at delivery diastolic (mm/Hg)	65.6 ± 11.4	111.4 ± 14.2	< 0.0001
Pulse rate (beats/min)	71.4 ± 1.6	up 70.8 ± 2.4	0.324
Proteinuria (g/day)	Nil	1.24 ± 0.86	/
Edema	Nil	++ in all cases	/

Endothelial dysfunction is considered to be a main cause of classical clinical features of pre – eclampsia (21).

Values are expressed as mean ± SD;
BP- blood pressure, P*= Two-Samples t-test probability

Table 2 depicts the concentrations of malondialdehyde and activities of various antioxidant enzymes in pair matched normotensive (control) group and preeclamptic maternal and cord blood. The MDA level in preeclamptic maternal blood was significantly high ($p<0.001$) compared to that of control. However its content in pre ec-

Table 2. Concentrations of malondialdehyde and activities of various antioxidant enzymes in pair-matched normotensive (Control) and preeclamptic maternal and cord blood

Parameters	Normotensive maternal blood (Group I) (n = 20)	Normotensive cord blood (Group II) (n = 20)	Preeclamptic maternal blood (Group III) (n = 20)	Preeclamptic cord blood (Group IV) (n = 20)	P*-value
Malondialdehyde (MDA) (mmol/gHb)	2.77± 0.361	1.716 ± 0.415	3.7879 ± 0.371	2.394 ± 0.36	<0.001
Superoxide dismutase (SOD) (IU/gHb)	587.8 ± 44.42	608.82 ± 40.88	497.17 ± 25.07	452.56 ± 24.68	<0.05
Glutathione peroxidase (GPx) (IU/gHb)	25.87 ± 1.653	27.53 ± 1.40	22.37 ± 1.03	21.19±1.13	<0.001
Glutathione reductase (GR) (IU/gHb)	8.94 ± 0.86	8.25 ± 0.78	7.35 ± 0.86	6.68±0.62	<0.001

lamptic cord blood compared to their pair matched blood was significantly low ($p<0.001$).
Values are expressed as mean ± SD. Mean and Standard Deviation were estimated for different variables in each study group. Mean values were compared between different study groups by using One way ANOVA

Several defense enzymes have been adopted by the erythrocyte to protect itself against the aggressive oxygen species by utilizing enzymatic and non enzymatic anti oxidants. Preeclamptic patients showed a decrease in activities of enzymatic antioxidants when compared to normotensive group. (Group I vs group III).

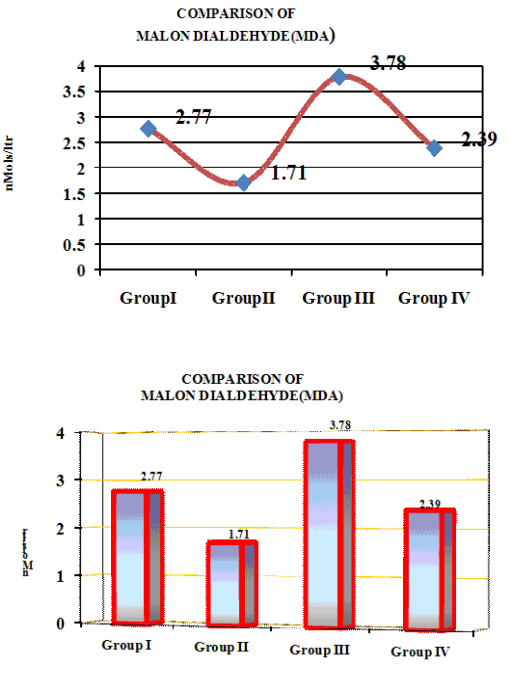
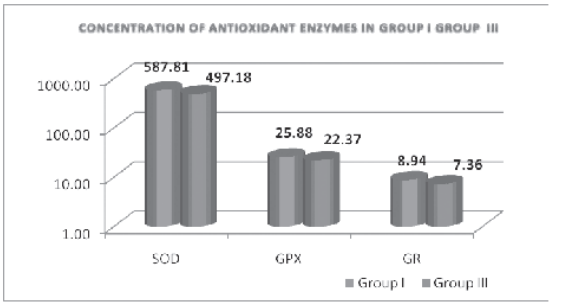


Figure 1. MDA concentrations in pair-matched normotensive and preeclamptic maternal and cord blood

The figure-1 depicts, MDA concentrations in pair-matched normotensive and preeclamptic maternal and cord blood. Increased lipid peroxidation products have been reported to be present in serum of pre eclamptic women. The byproducts of tissues lipid peroxidation propagate further lipid peroxidation in the same tissue and at sites distal to areas of initial damage. (22).

Figure 2. Concentration of antioxidant enzymes in normotensive and preeclamptic maternal blood.



The levels of enzymatic antioxidants in pair matched normotensive (control) group and pre eclamptic maternal and cord blood are depicted in Figure 2.

Preeclamptic patients had low levels of enzymatic antioxidants. The activity of Superoxide radical was significantly increased in cord blood ($p< 0.05$) of normotensive as compared to pair-matched maternal blood whereas in preeclamptic cord the activity of Superoxide radical was significantly decreased ($p< 0.05$) in comparison to pair-matched preeclamptic maternal blood. Levels of Glutathione peroxidase and Glutathione Reductase were significantly increased ($p< 0.001$) in normotensive cord blood and significantly decreased ($p< 0.001$) in preeclamptic cord blood compared to pair matched maternal blood. Figure 3 illustrated that Comparison of balance in biologic actions of antioxidants and lipid peroxides in normal pregnancy with imbalance of increased lipid peroxides and decreased vita-

min E in preeclamptic pregnancy. And finally figure 4 explains that pathophysiological changes that occur in Normal pregnancy compared with Preeclamsia

Figure 3 Comparison of balance in biologic actions of antioxidants and lipid peroxides in normal pregnancy with imbalance of increased lipid peroxides and decreased vitamin E in preeclamptic pregnancy. Wang *et al* (1991) December *Am. J.Obstet. Gynecol*

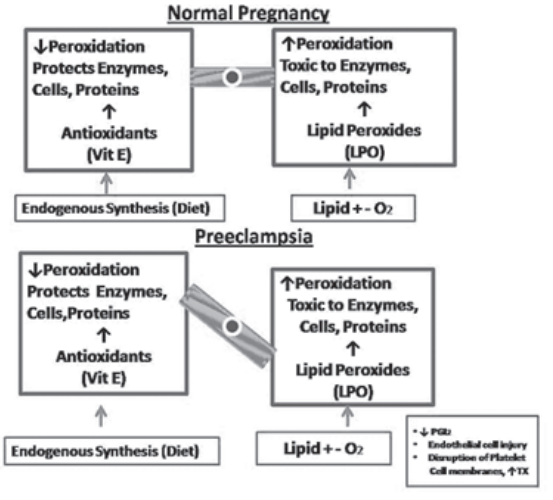
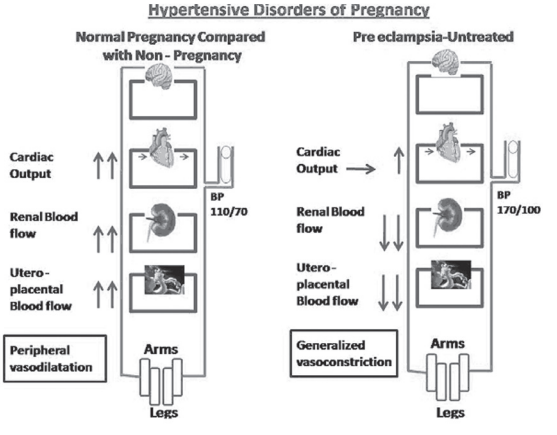


Figure 4 Hypothesized pathophysiological changes that may occur in Normal pregnancy compared with Preeclamsia



Discussion

There is an increasing evidence that oxidative stress may be an important contributing factor to the pathogenesis of pre-eclampsia (23-27). The pregnant women affected by pre-eclampsia may have abnormal ROS production, particularly NO and O_2, and abnormal levels of antioxidant defenses and increased placental lipid peroxidation (28). An imbalance between the pro–oxidants and the antioxidants has been defined as 'oxidative stress '. In severe pre-eclampsia, the balance between ROS and antioxidants is disturbed due to an increase in oxidants and compromise of antioxidants which is depicted in Figure 3.

Lipid peroxidation is a process that occurs normally at low levels in all cells and tissues. It involves conversion of unsaturated fatty acids to lipid hydroperoxides. This process can be initiated by free radicals, which are unstable molecules that possess an unpaired electron in their outer orbital. The organism normally has anti-oxidative mechanisms that limit this process. Moreover, low concentrations of lipid peroxides are essential and may act endogenously as intracellular messengers. Oxygen derived free radicals are produced as a result of metabolism of oxygen biradical during reduction reactions (29). Superoxide radicals are unique in that they can lead to the formation of many other reactive oxygen species including hydroxyl radicals. Superoxide radicals also reacts with Hydrogen peroxide to generate the singlet oxygen molecule (30). It is difficult to block the oxidative stress – induced injury to cells or tissues because ROS are continuously produced by cellular aerobic metabolism (31). Oxidative stress may be limited by using chain – breaking antioxidants such as vitamin E which neutralize hydroxyl, superoxide, and hydrogen peroxide radicals and prevents

oxidative stress (32). During normal human pregnancy, serum lipid peroxidation products are elevated (33) but are counterbalanced by and increased activity of the antioxidant system (34-36).Several studies suggest that pre-eclampsia is associated with increased circulating lipid peroxides compared to normal pregnancy (37-38).

The most in vivo source of oxygen derived free radical is molecular oxygen itself. The metabolism of oxygen (i.e reduction) generates ROS (39). Imbalance between the production of ROS and in vivo availability of antioxidants might play an indirect role in the etiology and complications of the disease (40). LPO formed at the primary site could be transferred through circulation and could provoke damage to internal organs (41). Hence LPO is a Biomarker for oxidative stress.

Antioxidant enzymes like SOD and glutathione peroxidase form the first line defense against ROS and decrease in their activities contributes to the oxidant assault on cells. Erythrocyte SOD is an important enzyme that specifically scavenges ROS – like superoxide radicals produced in the cells. GPx has been considered as a major protective enzyme against the accumulating organic peroxides (ROOH), which are potential radical forming species within the cell. GPx catalyzes the conversion of oxidized glutathione to reduced glutathione.Endogenous protective mechanisms against reactive oxygen species are enzymatic superoxide scavengers such as superoxide dismutase and glutathione peroxidase. In addition, there is an extensive non – enzymatic anti – oxidant network of different lipid – and water – soluble molecules that have in common the ability to scavenge free radicals. â – Carotene and vitamin E are two of the lipid – soluble substancesand ascorbic acid, uric acid and glutathione are some of the water – soluble free radical scavengers.

Lipid peroxidation products are candidate factors that may mediate disturbance of the maternal vascular endothelium (42). Glutathione peroxidase, an enzyme that removes hydrogen peroxide and converts lipid hydroperoxides to less reactive alcohols, may be deficient in placental tissue from preeclamptic women. This is seen in conjuction with increased *in vitro* placental production of lipid hydroperoxides and thromboxane A_2 (TXA_2) (43). TXA_2 is a vasoconstrictive and pro – aggregatory prostaglandin normally counterregulated by prostcyclin (PGI_2). Chemical inhibition of placental glutathione peroxidase resulted in increased production of lipid hydroperoxides and an increase in the placental TXA_2 to PGI_2 output ratio (44). Lipid hydroperoxides can inhibit PGI_2 synthase enzyme activity and simultaneously stimulate the cyclooxygenase component of PGH synthase. (45) Whereas TXA_2 synthase activity is unchanged or even stimulated (43, 46). Since expression of the synthases is not altered in the uteroplacental unit (47), these effects of lipid hydroperoxides could be the source of the decreased placental PGI_2 to TXA_2 production ratio in preeclampsia. The altered prostaglandin ratio might provoke vasospasm with exacerbation of placental ischemia, increased cell damage, and increased lipid peroxidation (amplification of oxidative stress) (48). The pathophysiological changes that occur in normal pregnancy compared with preeclamsia is depicted in Figure 4.

The present study investigated maternal plasma enzymatic antioxidant levels during normal and complicated preeclamptic pregnancies and their relationships with neonatal cord blood antioxidant levels. Cumulative evidences in recent years show that a biochemical imbalance in preeclampsia occurs with an increase of oxidative stress and a

deficient antioxidant protection (49) Indeed, free radicals released from the poorly perfused fetoplacental unit initiate lipid peroxidation by attacking polyunsaturated fatty acids in cell membranes, converting them to lipid peroxides and to a variety of antioxidant enzymes in preeclamptic cord blood which subsequently stimulate membrane phopholipid peroxidation by alkoxyl radicals (50). This study offers an opportunity to observe that oxidative stress is increased as the severity of the disease increases. From the findings of significantly low MDA contents in the pair-matched cord blood, it is hypothesized that antioxidant capacity of cord blood is sufficient and placental barrier is adequate, to shield the fetus from the oxidative injury.

Conclusion

To conclude, the oxidative stress status is low in the blood of neonates compared to its level in the pair-matched preeclamptic mothers, and oxidative stress status is increased in preeclamptic mothers compared to normotensive mothers. Further studies are needed to explore strategies so that the normal levels of antioxidant vitamins are maintained to combat preeclampsia in women at high risk. Hence it is inferred that antioxidant supplementation could prevent oxidative stress mediated changes to cell and biomolecules during the gestational period and further work is in progress in this direction.

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Plasmid mediated Naphthalene Degradation in *Pseudomonas* sp. strain NGK 1

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Abstract
A large indigenous plasmid was found in the naphthalene degrading *Pseudomonas* sp. (NGK1). This plasmid, pND15 appears to contain complete genetic information for the degradation of naphthalene as the plasmid cured strain failed to utilize naphthalene as source of carbon. The *E. coli* transformed with pND15 showed ability to degrade naphthalene and growth pattern similar to that of wild type *Pseudomonas* sp. (NGK1).

Key words: *Pseudomonas* sp. NGK1, Plasmid, Naphthalene degradation

Introduction

Polycyclic aromatic hydrocarbons (PAHs) such as naphthalene show potential toxicity to higher organisms (1-3). Owing to the bioaccumulation, inferred recalcitrance and genotoxicity, studies on the fate of naphthalene in the environment have acquired lot of importance. Attempts have also been made to find safe and reliable methods of disposing naphthalene wastes. Bioremediation is found to be the most acceptable and suitable method of decontaminating the polluted environments. However this technology mainly depends on isolation of the biological agents that either degrade or use the genobiotics as source of carbon and energy. Soil bacteria show immense potential of degrading the variety of recalcitrant chemicals that are found to be toxic to other forms of life. Naphthalene degrading soil bacteria that belong to different taxonomic groups have been isolated from polluted soil samples (4-13). In most of the cases indigenous plasmids have been implicated in the mineralization of naphthalene. These naphthalene degrading plasmids show considerable genetic diversity despite of having highly conserved naphthalene degrading genes (14-21). We have isolated naphthalene degrading *Pseudomonas* sp. NGK1 (10) from the effluent canals of a textile industry. This bacterial strain showed higher degradative efficiency and more tolerance to naphthalene than many naphthalene degrading strains reported till date. Therefore in the present study we attempted to understand the molecular basis of naphthalene degradation in *Pseudomonas* sp. (NGK1).

Materials and Methods

Media and growth conditions*:* The minimal-mineral salts medium contained (gm/L) K_2HPO_4 (0.38), $MgSO_4.7H_2O$ (0.2), KNO_3 (1.0), and $FeCl_3$ (0.05). The pH was adjusted to 7 and the medium was supplemented with filter sterilized naphthalene (0.1% w/v). This medium was used for growth studies and naphthalene utilization. While isolating plasmid pND15 the cultures were grown in the medium containing (g/L) bacto-

tryptone (10), yeast extract (5) and NaCl (5). The pH of the medium was adjusted to 7.5. When necessary 20 µg/mL mytomycin C was supplemented to the medium.

The minimal- mineral salts medium used for culturing *Escherichia coli* HB101contained filter sterilized proline (166 mg/L), thiamine (0.166 mg/L) and leucine (41 mg/L). When required minimal medium plates were prepared by adding 2% agar to the minimal salts medium.

The *Pseudomonas* sp. NGK1 culture was grown in the mineral salts medium supplemented with naphthalene (1%) as the sole source of carbon and energy. The growth of the bacterium was measured spectrophotometrically by monitoring the optical density at 660 nm at different incubation periods. Further cells were withdrawn from the 100 µl of the culture medium at different time intervals and the viable-cell population was counted by plating on nutrient-agar plates after performing the serial dilution. *Detection of plasmid DNA*: A single colony of *Pseudomonas* sp. NGK1 was taken from naphthalene mineral salts agar to inoculate 10 mL of LB medium and the culture was incubated for 18 h on a rotary shaker with vigorous shaking. Similarly *Flavobacterium* sp. ATCC27551 having indigenous plasmid pPDL2 (22) and *E. coli* cells without any plasmid were grown in LB medium. Plasmid preparations from these bacterial strains were isolated following the procedures described elsewhere (23) and analyzed on 0.8% agarose gel.

*Plasmid curing from Pseudomonas sp. NGK*1: The indigenous plasmid found in *Pseudomonas* sp. NGK1 is cured by using mytomycin C (24). The LB medium containing 20mg/mL of mytomycin C is inoculated with the over night culture of *Pseudomonas* sp. NGK1 and incubated for 18 h at 30°C. Serial dilutions were prepared from these cultures and plated on LB medium to develop single colonies. After formation of colonies, the individual colonies were patch plated on naphthalene - mineral salts agar plate. The colonies which failed to grow on naphthalene - mineral salts agar were selected from the master plate and examined for the presence of plasmid DNA.

Molecular size determination of plasmid: The E.coli stains containing plasmids of known molecular weights were grown in LB medium and plasmid preparations from those cultures were made and analyzed on 0.8% agarose gels along with the indigenous plasmid pND15 of Pseudomonas sp. NGK1 and its molecular weight was determined by following the procedure (24).

Transformation : The competent *Escherichia coli* HB101 cells and the plasmid cured *Pseudomonas* sp. NGK1, *Nah*$^-$, *Sal*$^-$ were prepared and transformed them with plasmid pND15 following the procedures described in Promega protocol and application guide (25). The transformants were selected by plating on naphthalene/salicylate mineral salts agar plates amended with filter sterilized proline, thiamine and leucine. The colonies were then sub-cultured and were screened for the presence of plasmid DNA following the methods described elsewhere.

Growth Behaviour of transformed Escherichia coli HB101 : The growth behaviour of transformed *Escherichia coli* HB101 was carried out by incubating actively growing culture (5% v/v) into 250 mL Erlenmeyer flask containing 100 mL mineral salts medium amended with naphthalene (0.1% w/v), filter sterilized proline (166 mg/L),thiamine (0.166 mg/L) and leucine (41 mg/L). The culture was incubated on a rotary

shaker and the growth of the *E.coli* cells with and without plasmid pND15 was measured.

Enzyme assays: The cell free extract was prepared from freshly grown cells of Pseudomonas sp. NGK1 and transformed Escherichia coli HB101. The obtained cell free extract was used for different enzymatic assays. Naphthalene 1, 2- dioxygenase, 1, 2-dihydroxynaphthalene dioxygenase, salicylate hydroxylase, catechol 1, 2 dioxygenase and catechol 2, 3 -dioxygenase were assayed according to the methods described elsewhere (7). The concentration of the protein in the cell free extract was determined spectroscopically (26). The specific activity of enzyme was expressed as µmol substrate converted or product formed/min/mg of protein.

Results and Discussion

A naphthalene degrading *Pseudomonas* sp. strain NGK1 was used to study its ability to utilize naphthalene as a sole source of carbon and energy. The growth behaviour of the bacterium is presented in fig. 2. It was shown that the growth of the bacterium was increased with increase in the incubation time and after 18 h the growth entered the stationary phase. The initial viable cell population of $2x10^6$ cfu/mL has increased to $5x10^{10}$ cfu/mL after 24 h of incubation in the mineral salt medium supplemented with naphthalene (0.1% w/v). To gain better insight into the molecular mechanism of naphthalene degradation in *Pseudomonas* sp. strain NGK 1, an attempt was made to detect the presence of indigenous plasmid in this bacterium. While attempting to isolate the plasmid from *Pseudomonas* sp. strain NGK 1. We have also used *Flavobacterium* sp. ATCC 27551 having well characterized indigenous plasmid as positive control and plasmid less *E. coli* HB101 as negative control. The plasmid DNA was found only in *Pseudomonas* sp.NGK1 and *Flavobacterium* sp. ATCC 27551 (Fig. 1) whereas no plasmid band was found in plasmid preparations made from plasmid less *E.coli* cells. These results clearly indicate existence of indigenous plasmid in *Pseudomonas* sp. NGK1 and it is named as pND15. Subsequently, the

Fig 1. Plasmid preparations from *E.coli* HB101 (lane 1) and *Pseudomonas* sp.NGK1 (lane 2). Large indigenous plasmid is found in lane 2

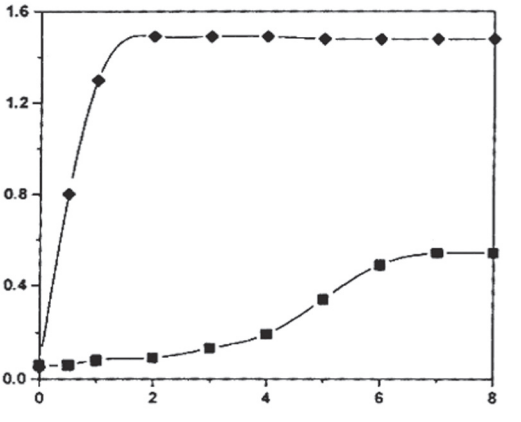

Fig 2. Growth behaviour of *Pseudomonas* sp. strain NGK1 (-♦-) and plasmid pND15 transformed *Escherichia coli* (-¦-) in the mineral salts medium containing naphthalene (7.8 mM) as the sole source of carbon.

involvement of pND15 in the degradation of naphthalene is assessed. The plasmid DNA from *Pseudomonas* sp. NGK1 was cured by using mytomycin C and the cured strain was tested for its ability to grow on naphthalene containing medium. Evidently the plasmid cured strain could not grow on naphthalene containing minimal medium indicating the presence of naphthalene degrading genes on plasmid pND15. Further, the cell free extracts prepared from this plasmid cured strain showed no naphthalene degrading enzyme activities that are otherwise found in the wild type strain of *Pseudomonas* sp. This further strengthens the involvement of pND15 in the degradation of naphthalene. Similarly the *E.coli* cells transformed with pND15 showed the presence of complete set of enzymes involved in naphthalene degradation. Interestingly the *E.coli* HB101 cells having plasmid pND15 and wild type *Pseudomonas* sp. NGK1 have shown similar growth pattern (Fig.2). Several naphthalene-degrading plasmids were found in *Pseudomonas* strains isolated from diversified geographical regions. These plasmids show maximum genetic diversity despite of having highly conserved naphthalene degrading genes (16). In most of the cases the size of the plasmid along with other characteristics such as restriction pattern, hybridization profile is taken as an important feature to draw the comparisons among various degradative plasmids. Therefore the molecular size of pND15 is determined; from this the size of the plasmid pND15 was determined to be approximately 150Kb. In order to assess the similarity of the pND15 with other naphthalene degrading plasmids the plasmid pND15 was digested with several restriction endonucleases such as *Eco*RI, *Pst*I, *Bam* HI, *Bgl* II, *Hind* III and the restriction pattern thus obtained was compared with the restriction maps of known naphthalene degrading plasmids (Fig 3). Surprisingly none of the known naphthalene degrading plasmids shows restriction profile that matches with the restriction pattern of pND15.

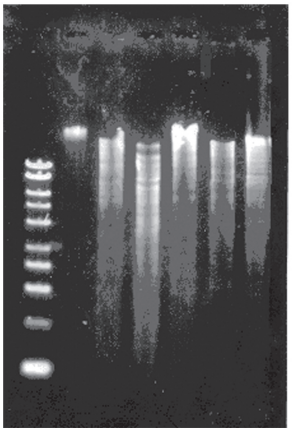

Fig 3. Restriction of pattern of plasmid pND15. Lane 1, Molecular Weight markers, Lane 2 represents uncut plasmid pND15, Lane 3 to 1 are digests of pND15 with *Eco*RI, *Pst*I, *Bam* HI *Bgl* II, *Hind* III

The plasmid pND15, when transformed into *E.coli* encodes necessary enzymes for degrading naphthalene (Table 1). It was observed that the

Sl.No.	Enzyme	*Pseudomonas* sp.	*Escherichia coli* HB101
1.	NDO	0.99	0.12
2.	1, 2 – DHND	4.76	0.30
3.	SALH	0.60	0.09
4.	C23O	0.48	0.10
5.	C12O	0.10	0.08

Table 1. Activities of various enzymes in the crude cell free extracts of the cells of *Pseudomonas* sp. strain NGK1 and *Escherichia coli*(pND15) grown in the mineral salts medium containing naphthalene.

Enzyme activity: µmol substrate converted or product formed $min^{-1}mg^{-1}$ protein.
NDO- (Naphthalene-1, 2-dioxygenase), 1, 2-DHND- (1, 2-dihydroxynaphthalene dehydrogenase), SALH-(Salicylate hydroxylase), C23O-(Catechol –2, 3-dioxygenase) and C12O- (Catechol 1, 2-dioxygenase).

growth of *E.coli* in the mineral salts medium showed an initial lag phase and then entered into the exponential phase that lasted for another 50 h. The viable-cell population from 10^7 cfu/ml increased to 10^9 cfu/mL after 120 h incubation. Further the complete degradation of naphthalene was observed at 140 h incubation. The cell free extracts of *Escherichia coli* HB101 also showed the accumulation of α- hydroxymuconic semialdehyde. The enzyme activities of various enzymes involved in naphthalene degradation were found in the cell free extracts of *Pseudomonas* sp. strain NGK1 and transformed *Escherichia coli*. These results clearly demonstrate the existence of complete genetic mechanism for degradation of naphthalene on indigenous plasmid pND15. Further, we have tested the wild type strain of *Pseudomonas* sp. NGK1 to assess its ability to degrade several other monocyclic, dicyclic and substituted hydrocarbons. This strain besides efficiently degrading anthracene and phenanthrene shows high tolerance towards naphthalene and hence can be a good candidate to treat the industrial effluents containing high concentrations of aromatic hydrocarbons.

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Synthesis and anti-microbial screening of some new 6,7,8,9-Tetrahydro-5*H*-5-(2'-hydroxyphenyl)-2-(4'-substitutedbenzylidine)-3-(4-nitrophenylamino) thiazoloquinazoline derivatives

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Abstract
A series of some new 6,7,8,9-Tetrahydro-5*H*-5-(2'-hydroxyphenyl)-2-(4'-fluorobenzylidine)-3-(4-nitrophenyl amino) thiazolo quinazoline (4a-4d) and 6,7,8,9-Tetrahydro-5*H*-5-(2'-hydroxy phenyl)-2-(4'-substituted benzylidine)-3-(4-nitrophenyl amino) thiazolo quinazoline (5a-5d) derivatives were synthesized. The *in vitro* anti-bacterial and anti-fungal activities were determined by paper disk diffusion method. The minimum inhibitory concentrations (MIC) of the compounds were also determined by agar streak dilution method. Most of the synthesized compounds exhibited significant anti-bacterial and anti-fungal activities. Among the synthesized compounds 6,7,8,9-Tetra hydro-5*H*-5-(2'-hydroxy phenyl)-2-(4'-methoxy benzylidine)-3-(4-nitro phenyl amino)thiazolo quinazoline 5a, 6,7,8,9-Tetrahydro-5*H*-5-(2'-hydroxy phenyl)-2-(3',4'-dimethyl benzylidine)-3-(4-nitrophenyl amino) thiazolo quinazoline 5b and 6,7,8,9-Tetrahydro-5*H*-5-(2'-hydroxyphenyl)-2-(4'-fluoro benzylidine)-3-(4-nitro phenylamino) thiazolo quinazoline 5d exhibited most potent *in vitro* antimicrobial activity with MIC of (9.4, 10.2, 10.4, 10.2, 11.3, 12.1, 12.7, 14.6, 18.7), (10.3, 14.2, 11.6, 13.4, 13.6, 13.8, 14.6, 13.1, 14.5) and (12.5, 10.3, 14.1, 10.6, 13.4, 19.8, 17.8, 11.2, 13.6) at 100 μg mL^{-1} against *Staphylococcus aureus, Staphylococcus epidermidis, Micrococcus luteus*, *Bacillus cereus, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Aspergillus niger* and *Candida albicans.*

Key words: Thiazoloquinazoline, Benzylidine thiazoloquinazoline, Nitrophenyl amino Thiazoloquinazoline, Dimethyl group substitution, anti-bacterial, anti-fungal.

Introduction

Bacterial infections such as food poisoning, rheumatic, salmonellosis and diarrhea are caused by multidrug-resistant Gram -positive and Gram-negative pathogens. Principal players among these problematic organisms are isolates of methicillin resistant Staphylococcus aureus, Staphylococcus pyogenes, Salmonella typhimurium and Escherichia coli. Million of people in the subtropical regions of the world are infected and 20,000 deaths every year due to these parasitic bacterial infections. Amoxicillin, norfoxacin, ciprofoxacin are the principal drugs of choice in the treatment of bacterial infect ion since they are effective against extra intestinal and intestinal wall infection, but these are associated with several side effects such as nausea, metallic taste, dizziness, hypertension, etc. as well as resistance have been reported. The present strategy for new drug development is directed towards identifying the essential enzyme system in the bacterial and developing molecules to in-

hibit them on our going medicinal chemistry research activity. We have found that quinazolines and condensed quinazolines are classes of fused heterocycles that are of considerable interest because of the diverse range of their biological properties. Among a wide variety of nitrogen heterocycles that have been explored for developing pharmaceutically important molecules. Many derivatives of quinazoline are used in the pharmaceutical industry, in medicine and in agriculture due to their antimicrobial (1-3), anti-infammatory (4, 5), diuretic (6), anti-convulsant (7), anti-allergic (8), anti-hypertensive (9, 10) and anti-parkinsonism efects (11). Some of the quinazolines induced mutagenic activity (12,13). As documented in the literature, many quinazolines act as anticancer active agents (14-17). Our analogue-based design encompasses the synthesis of 6,7,8,9-Tetra hydro-5H-5-(2'-hydroxy phenyl)-2-(4'-substituted benzylidine)-3-(4-nitrophenylamino)thiazoloquinazoline derivatives intended to be tested for their *in vitro* antimicrobial properties against Gram positive and Gram negative bacteria and fungi.

Materials and Methods

Materials: Synthetic starting compounds, reagents and solvents were of analytical reagent grade or of the highest quality commercially available and were purchased from Aldrich Chemical Co., Merck Chemical Co. and were dried when necessary. The melting points were taken in open capillary tube and are uncorrected. IR spectra were recorded with KBr pellets (ABB Bomem FT-IR spectrometer MB 104 ABB Limited, Bangaluru, India). Proton (^{1}H) NMR spectra (Bruker 400 NMR spectrometer) were recorded with TMS as internal standard. Mass spectral data were recorded with a quadrupol mass spectrometer (Shimadzu GC MS QP 5000), and microanalyses were performed using a *vario EL* V300 *elemental* analyzer (Elemental Analysensysteme). The purity of the compounds was checked by TLC on pre-coated silica gel (HF_{254}, 200 mesh) aluminium plates (E.Merck) using ethyl acetate: benzene (1:3) and visualized in UV chamber. IR, ^{1}H-NMR, mass spectral datas and elemental analysis were consistent with the assigned structures.

Experimental methods: The synthetic strategy leading to the key intermediate and the target compounds are illustrated in Scheme. 6,7,8,9-Tetrahydro-5*H*-5-(2'-hydroxyphenyl)-thiazolo (2,3-*b*) quinazolin-3(2*H*)-one 3 prepared by taking the equimolar quantities of each (0.039 mol) of cyclohexanone and salicylaldehyde (0.039 mol) then it in a beaker, to this sodium hydroxide solution was added to make the solution alkaline, this was shaken and kept aside. The solid thus obtained, was filtered, washed with water and recrystallized from absolute ethanol. A mixture of 2-Hydroxybenzylidine cyclohexanone 1 (0.039 mol) thiourea (0.03 mol) and potassium hydroxide (2.5g) in ethanol (100 ml) was heated under reflux for 3h. The reaction mixture was concentrated to half of its volume, diluted with water, then acidified with dilute acetic acid and kept overnight. The solid thus obtained was filtered, washed with water and recrystallized from ethanol to give 4-hydroxyphenyl 3,4,5,6,7,8-Hexahydro quinazolin-2-thione 2. The chloroacetic acid (0.096 mol) was melted on a water bath and thione (0.009 mol) added to it portion wise to maintain its homogeneity. The homogeneous mixture was further heated on a water bath for 30 min and kept overnight. The solid thus obtained was washed with water and crystallized from ethanol to give 6,7,8,9-Tetrahydro-5*H*-5-(2'-hydroxy phenyl) thiazolo (2, 3-*b*) quinazolin-3-(2*H*)-one 3 (18).

A mixture of 3 (0.002 mol), substituted benzaldehyde (0.002 mol) and anhydrous sodium acetate (0.002 mol) in glacial acetic acid (10 ml)

was heated under reflux for 4h. The reaction mixture was kept overnight and the solid, thus separated was filtered, washed with water and recrystallized from ethanol to furnish 6,7,8,9-Tetrahydro-5*H*-5-(2'-hydroxyphenyl)-2-(4'-substituted benzylidine) thiazolo (2,3-*b*) quinazolin-3-(2*H*)-one (4a-4d). Equimolar quantities (0.004 mol) of compounds 4a-4d treated with thionyl chloride and DMF to get chloro derivative and then coupled with *p*-nitroaniline in DMF at 80^{0}C and quenched in ice-water to get the products, which were separated by filtration, vaccum dried and recrystallized from warm ethanol to yield 6,7,8,9-Tetrahydro-5*H*-5-(2'-hydroxy phenyl)-2-(4'-substituted benzylidine)-3-(4-nitrophenyl amino) thiazolo quinazolines (5a-5d). The spectral data IR, ^{1}H NMR, mass spectroscopy and elemental analyses were used to ascertain the structures of all the compounds.

^{1}H NMR spectra were recorded for all the target compounds. The ^{1}H NMR spectra were recorded for the representative key intermediate 3. The 6,7,8,9-Tetrahydro-5*H*-5-(2'-hydroxy phenyl) thiazolo (2, 3-*b*) quinazolin-3-(2*H*)-one. Yield: 71%; m.p.153-155°C, IR (KBr, cm^{-1}): 3402 (phenolic OH), 3046 (Ar-CH), 1719 (C=O), 1462 (C=C) cm^{-1}; ^{1}H-NMR ($CDCl_3$) ä: 9.91 (s, 1H; Ar-OH), 6.61-6.89 (m,4H Ar-H), 5.71 (s, 1H; -CH) 3.76 (s, 2H; -CH_2) 1.6-2.42 (m, 8H; 4XCH_2).EI-MS m/z [M]+: 300 (Calcd for $C_{16}H_{16}N_2O_2S$; 300.38). Anal. Calcd for $C_{16}H_{16}N_2O_2S$; C, 63.98; H, 5.37; N, 9.32; Found: C, 63.92; H, 5.28; N, 9.30.

6,7,8,9-Tetrahydro-5*H*-5-(2'-hydroxyphenyl)-2-(4'-methoxybenzylidine) thiazolo(2,3-*b*) quinazolin-3-(2*H*)-one (4a): Pale yellow solid; Yield: 78%; mp. 183-185°C, IR : 3476 (O-H), 3096 (Ar-CH), 1728 (C=O), 1468 (C=C) cm^{-1}. ^{1}H-NMR ($CDCl_3$): ä 9.84 (s, 1H, Ar-OH), 6.96-7.54 (m, 8H, Ar-H), 6.67 (s, 1H, =CH), 5.83 (s, 1H, H-5), 3.75 (s, 3H –OCH_3), 1.58-2.62 (m, 8H, 4 × CH_2); EI-MS (m/z, %): 418 [M]+; (Calcd for $C_{24}H_{22}N_2O_3S$; 418.51). Anal. Calcd for $C_{24}H_{22}N_2O_3S$, C, 68.88; H, 5.30; N, 6.69; Found: C, 68.90; H, 5.33; N, 6.72.

6,7,8,9-Tetrahydro-5*H*-5-(2'-hydroxyphenyl) -2-(3',4'-dimethylbenzylidine) thiazolo(2,3-*b*) quinazolin-3-(2*H*)-one (4b): Solid; Yield: 79%; mp. 181-183 °C, IR : 3450 (O-H), 3051 (Ar-CH), 1724 (C=O), 1437 (C=C) cm^{-1}; ^{1}H-NMR ($CDCl_3$): ä 6.89-7.76 (m, 7H, Ar-H), 6.74 (s, 1H, =CH), 5.78 (s, 1H, H-5), 9.84 (s, 1H, Ar-OH), 2.23 (s, 6H 2×CH_3), 1.62-2.32 (m, 8H, 4×CH_2); EI-MS (m/z, %): 416 [M]+; (Calcd for $C_{25}H_{24}N_2O_2S$; 416.16). Anal. Calcd for $C_{25}H_{24}N_2O_2S$; C, 72.09; H, 5.81; N, 6.73; Found: C, 72.12; H, 5.79; N, 6.75.

6,7,8,9-Tetrahydro-5*H*-5-(2'-hydroxyphenyl)-2-(4'-methylbenzylidine) thiazolo(2,3-*b*) quinazolin-3-(2*H*)-one (4c): Cream solid; Yield: 76%; mp. 186-188°C, IR : 3448 (O-H), 3049 (Ar-CH), 1721 (C=O), 1434 (C=C) cm^{-1}; ^{1}H-NMR ($CDCl_3$): ä 6.86-7.74 (m, 8H, Ar-H), 6.72 (s, 1H, =CH), 5.76 (s, 1H, H-5), 9.76 (s, 1H, Ar-OH), 2.20 (s, 3H –CH_3), 1.62-2.32 (m, 8H, 4 × CH_2); EI-MS (m/z, %): [M]+ 402; (Calcd for $C_{24}H_{22}N_2O_2S$; 402.14). Anal. Calcd for $C_{24}H_{22}N_2O_2S$; C, 71.00; H, 5.51; N, 6.96; Found: C, 69.87; H, 5.32; N, 6.74.

6,7,8,9-Tetrahydro-5*H*-5-(2'-hydroxyphenyl)-2-(4'-fluorobenzylidine) thiazolo(2,3-*b*) quinazolin-3(2*H*)-one (4d). Pale yellow solid; Yield: 69%; mp. 153-155°C, IR : 3437 (O-H), 3026 (Ar-CH), 1729 (C=O), 1522 (C=C), 826 (C-F) cm^{-1}; ^{1}H-NMR ($CDCl_3$): ä 9.94 (s, 1H, Ar-OH),6.63-7.32 (m, 8H, Ar-H), 6.38 (s, 1H, =CH), 5.87 (s, 1H, H-5), 1.34-2.33 (m, 8H, 4 × CH_2); EI-MS (m/z, %): [M]+406; (Calcd for $C_{23}H_{19}FN_2O_2S$; 406.12). Anal. Calcd

for $C_{23}H_{19}FN_2O_2S$; C, 67.96; H, 4.71; N, 6.89; Found: C, 67.97; H, 4.73; N, 6.87.

6,7,8,9-Tetrahydro-5*H*-5-(2'-hydroxyphenyl) -2-(4'-methoxybenzylidine)-3-(4-nitrophenyl amino)thiazoloquinazoline (5a): Pale yellow solid; Yield: 76%; mp. 156-158°C, IR : 3464 (O-H), 3027 (Ar-CH), 1494 (C=C), 1306 (N-H bending), 3396 (N-H stretching) cm^{-1}; ^{1}H-NMR ($CDCl_3$): ä 9.87 (s, 1H, Ar-OH), 6.72-7.23 (m, 12H, Ar-H), 6.36 (s, 1H, =CH),5.62 (s, 1H, H-5), 4.46 (s, 1H, thiazole), 3.78 (s, 3H –OCH_3), 7.26 (s, 1H, N-H), 1.46-2.42 (m, 8H, 4 × CH_2); EI-MS (m/z, %): [M]+ 540; (Calcd for $C_{30}H_{28}N_4O_4S$; 540.18). Anal. Calcd for $C_{30}H_{28}N_4O_4S$; C, 66.65; H, 5.22; N, 10.36; Found: C, 66.67; H, 5.25; N, 10.38.

6,7,8,9-Tetrahydro-5*H*-5-(2'-hydroxyphenyl) -2-(3',4'-dimethylbenzylidine)-3-(4-nitrophenyl amino)thiazoloquinazoline (5b): Yellow solid; Yield: 77%; mp. 181-183°C, IR : 3429 (O-H), 3027 (Ar-CH), 1413 (C=C), 1334 (N-H bending), 3313 (N-H stretching) cm^{-1}; ^{1}H-NMR ($CDCl_3$): ä 9.93 (s, 1H, Ar-OH), 6.79-7.24 (m, 12H, Ar-H), 6.26 (s, 1H, =CH), 5.74 (s, 1H, H-5), 4.39 (s, 1H, thiazole), 2.34 (s, 6H, 2XCH_3), 7.62 (s, 1H, N-H), 1.36-2.41 (m, 8H, 4 × CH_2); EI-MS (m/z, %): [M]+ 538; (Calcd for $C_{31}H_{31}N_4O_3S$; 538.2). Anal. Calcd for $C_{31}H_{31}N_4O_3S$; C, 69.12; H, 5.61; N, 10.40; Found: C, 69.14; H, 5.63; N, 10.43.

6,7,8,9-Tetrahydro-5*H*-5-(2'-hydroxyphenyl) -2-(4'-methylbenzylidine)-3-(4-nitrophenyl amino)thiazoloquinazoline (5c): Cream solid; Yield: 76%; mp. 193-192°C, IR : 3438 (O-H), 3024 (Ar-CH), 1412 (C=C), 1322 (N-H bending), 3310 (N-H stretching) cm^{-1}; ^{1}H-NMR ($CDCl_3$): ä 9.82 (s, 1H, Ar-OH), 6.69-7.24 (m, 12H, Ar-H), 6.28 (s, 1H, =CH), 5.72 (s, 1H, H-5), 4.45 (s, 1H, thiazole), 2.28 (s, 3H, –CH_3), 7.69 (s, 1H, N-H), 1.36-2.41 (m, 8H, 4 × CH_2); EI-MS (m/z, %): [M]+ 524; (Calcd for $C_{30}H_{28}N_4O_3S$; 524.19). Anal. Calcd for $C_{30}H_{28}N_4O_3S$; C, 68.68; H, 5.38; N, 10.68; Found: C, 68.65; H, 5.36; N, 10.70.

6,7,8,9-Tetrahydro-5*H*-5-(2'-hydroxyphenyl) -2-(4'-fluorobenzylidine)-3-(4-nitrophenyl amino)thiazoloquinazoline (5d): Cream solid; Yield: 89%; mp. 184-186°C, IR : 3449 (O-H), 3026 (Ar-CH), 1524 (C=C), 1316 (N-H bending), 3319 (N-H stretching), 821 (C-F) cm^{-1}; ^{1}H-NMR ($CDCl_3$): ä 9.96 (s, 1H, Ar-OH), 6.74-7.32 (m, 12H, Ar-H), 6.23 (s, 1H, =CH), 5.84 (s, 1H, H-5), 4.42 (s, 1H, thiazole), 7.34 (s,1H, N-H), 1.24-2.32 (m, 8H, 4 × CH_2); EI-MS (m/z, %): [M]+ 528; (Calcd for $C_{29}H_{25}FN_4O_3S$; 528.16). Anal. Calcd for $C_{29}H_{25}FN_4O_3S$; C, 65.89; H, 4.77; N, 10.60; Found: C, 65.91; H, 4.79; N, 10.62.

Antimicrobial Screening: All the synthesized compounds were screened for anti-bacterial and anti-fungal activities by paper disc diffusion technique. The anti-bacterial activity of the compounds were evaluated against four gram positive bacteria (*Staphylococcus aureus* ATCC 9144*, Staphylococcus epidermidis* ATCC 155*, Micrococcus luteus* ATCC 4698 and *Bacillus cereus* ATCC 11778) and three gram negative bacteria (*Escherichia coli* ATCC 25922*, Pseudomonas aeruginosa* ATCC 2853, *and Klebsiella pneumoniae* ATCC 11298*)*. The anti-fungal activities of the synthesized compounds were evaluated against two fungi (*Aspergillus niger* ATCC 9029 and *Candida albicans* ATCC 2091). The observed data on the antimicrobial activity of the synthesized compounds and standard drugs are given in Table 1.

Paper Disc Diffusion Technique: The sterilized (19) (autoclaved at 120°C for 30 minutes) medium (40-50°C) was inoculated (1 mL/100 mL of medium) with the suspension (10^5 cfu mL^{-1})

of the microorganism (matched to McFarland barium sulphate standard) and poured into a petridish to give a depth of 3-4 mm. The paper impregnated with the test compounds (μg mL^{-1} in dimethyl formamide) was placed on the solidified medium. The plates were pre-incubated for 1 h at room temperature and incubated at 37°C for 24 and 48 h for anti-bacterial and anti-fungal activities, respectively. Ciprofloxacin (Dr. Reddy's Laboratories, Batch No: IC666E04, India) and Ketoconazole (Wuhan Shengmao Corporation, Batch No: SBML/403, China) were used as standard for anti-bacterial and anti-fungal activities, respectively. The observed zone of inhibition is presented in table-1 and figures.

Minimum Inhibitory Concentration (MIC): MIC (20) of the compound was determined by agar streak dilution method. A stock solution of the synthesized compound (100 μg mL^{-1}) in dimethylformamide was prepared and graded quantities of the test compounds were incorporated in specified quantity of molten sterile agar (nutrient agar for anti-bacterial activity and sabouraud dextrose agar medium for antifungal activity). A specified quantity of the medium (40-50°C) containing the compound was poured into a petridish to give a depth of 3-4 mm and allowed to solidify. Suspension of the microorganism were prepared to contain approximately 10^5 cfu mL^{-1} and applied to plates with serially diluted compounds in dimethylformamide to be tested and incubated at 37°C for 24 h and 48 h for bacteria and fungi, respectively. The MIC was considered to be the lowest concentration of the test substance exhibiting no visible growth of bacteria or fungi on the plate. The observed MIC is presented in table-1.

Statistical Analysis: Student's *t*-test was used to determine a significant difference between the control.

Results and Discussion

The synthesized series of heterocycles, 4a-4d and 5a-5d by the reaction of 3 with appropriate aromatic aldehydes and *p* –nitro aniline in the presence of anhydrous sodium acetate and DMF as presented in Scheme. The IR, ^{1}H-NMR, mass spectroscopy and elemental analysis for the new compound is in accordance with the assigned structures. The IR spectra of compounds 4a-4d showed stretching bands of keto group at 1715-1740 cm^{-1}. In 5a-5d stretching and bending NH bands of thiazolo quinazoline moiety appear at 3300-3400 cm^{-1}, 1300-1350 cm^{-1} respectively. The recorded IR spectra of representative compounds 5a-5d showed missing of keto group bands. This clearly envisages that the keto group of 4a-4d is

Table 1. Anti-microbial activity of the synthesized compounds (100 μg/ml)

Compounds	*In vitro* activity - zone of inhibition (MIC)								
	S.aureus	S.epidermidis	M.luteus	B.cereus	E.coli	P.aeuriginosa	K.pneumoniae	A.niger	C.albicans
4a	15(23.6)	19(20.4)	15(26.7)	16(19.2)	18(24.9)	17(19.8)	14(19.6)	18(21.1)	19(16.6)
4b	19(19.3)	18(21.2)	15(16.3)	17(15.3)	18(19.2)	18(18.6)	15(22.9)	19(27.4)	16(19.9)
4c	17(13.2)	16(25.2)	17(20.6)	15(16.7)	17(19.0)	17(19.7)	17(16.6)	18(24.3)	17(26.6)
4d	18(13.6)	16(14.8)	18(12.4)	17(22.2)	16(14.4)	16(23.9)	19(13.8)	17(13.8)	18(14.2)
5a	24(9.4)	27(10.2)	22(10.4)	20(10.2)	23(11.3)	23(12.1)	26(12.7)	19(14.6)	19(18.7)
5b	22(10.3)	25(14.2)	24(11.6)	21(13.4)	25(13.6)	21(13.8)	25(14.6)	18(13.1)	18(14.5)
5c	21(10.4)	22(9.6)	23(11.2)	19(12.1)	21(14.8)	20(16.8)	20(13.9)	20(12.4)	19(16.6)
5d	19(12.5)	20(10.3)	19(14.1)	18(10.6)	19(13.4)	18(19.8)	16(17.8)	24(11.2)	22(13.6)
Ciprofloxacin	25	29	27	23	29	25	27	-	-
Ketoconazole	-	-	-	-	-	-	-	29	26
DMF	-	-	-	-	-	-	-	-	-

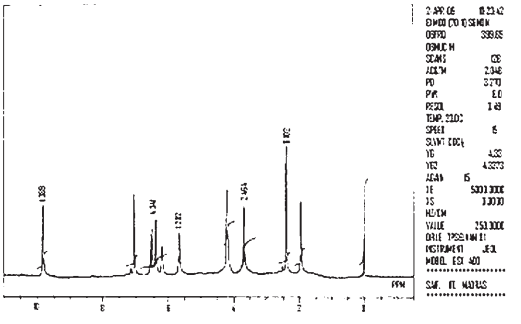


Figure 1 - The 6,7,8,9-Tetrahydro-5*H*-5-(2'-hydroxyphenyl)-thiazolo (2,3-*b*) quinazolin-3(2*H*)-one **3.**

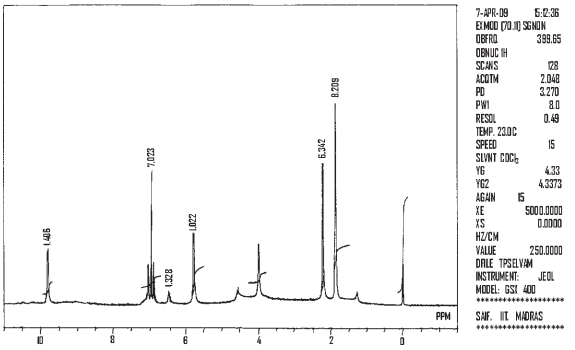


Figure 2 - 6,7,8,9-Tetra hydro-5*H*-5-(2'-hydroxy phenyl)-2-(3',4'-dimethyl benzylidine) thiazolo (2, 3-b) quinazolin-3-(2*H*)-one **4b.**

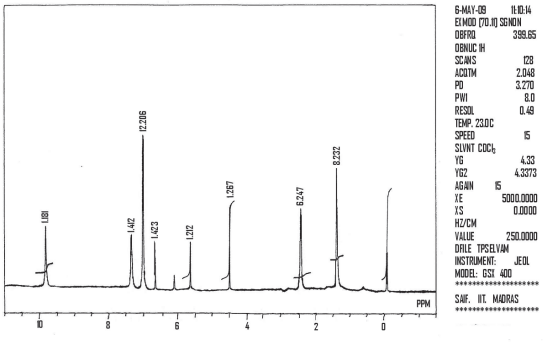


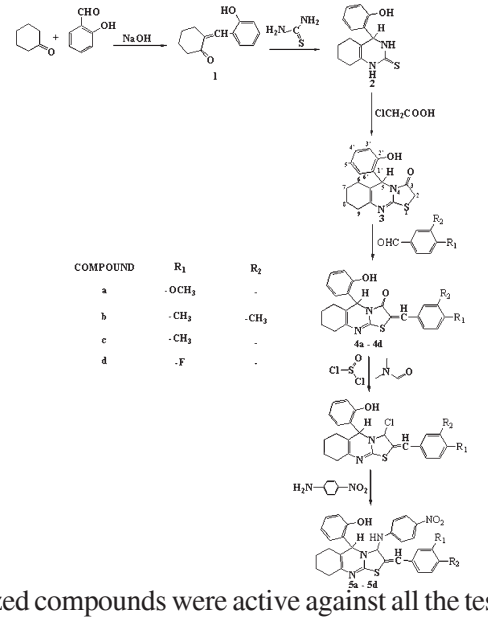
Figure 3- 6,7,8,9-Tetra hydro-5*H*-5-(2'-hydroxy phenyl)-2-(3',4'-dimethyl benzylidine)-3-(4- nitrophenyl amino) thiazolo quinazoline **5b.**

converted in to secondary NH. The proton magnetic resonance spectra of thiazoloquinazoline and their corresponding derivatives have been recorded in $CDCl_3$. In this 5a-5d NH signal of 3-(4-nitro phenyl) amino thiazolo quinazoline moiety appear at 7.26 (s), 7.62 (s), 7.69 (s), 7.34 (s) ppm respectively. The position and presence of NH signal in the ^{1}H-NMR spectra of final compounds conforms the secondary NH proton in thiazoloquinazoline moiety. This clearly envisages that thiazole-3-one moiety involve in 3-(4-nitro phenyl) amino formation. All these observed facts clearly demonstrate that 3rd position of keto group in thiazole ring is converted in to secondary amino group as indicated in figures- 1,2 and 3, it conforms the proposed structure 5a -5d.

All the synthesized compounds exhibited moderate to good anti-bacterial and anti-fungal activity. Among the synthesized compounds, compound 5a, 5b and 5d were found to possess significant anti-bacterial and anti-fungal activity when compared to standard drug Ciprofloxacin and Ketoconazole for anti-bacterial and anti-fungal activity respectively. Compound 5d displayed moderate anti-microbial activity where as the remaining compounds 4a, 4b, 4c and 4d shown lesser activity. The MIC of the synthesized compounds was established screened by agar streak dilution method with an MIC range of 9.4-27.4 μg mL^{-1}. 6,7,8,9-Tetrahydro-5*H*-5-(2'-hydroxyphenyl)-2-(4'-methoxy benzylidine)-3-(4-nitrophenyl amino) thiazoloquinazoline 5a and 6,7,8,9-Tetra hydro-5*H*-5-(2'-hydroxy phenyl)-2-(3',4'-dimethyl benzylidine)-3-(4-nitrophenyl amino) thiazolo quinazoline 5b was found to exhibit the highest anti-bacterial activity against *S.aureus* (9.4 μg mL^{-1}), *S.epidermidis* (9.6 μg mL^{-1}), *M.luteus* (10.4 μg mL^{-1}), *B.cereus* (‘10.2 μg mL^{-1}), *E.coli* (11.3 μg mL^{-1}), *P.aeruginosa* (12.1 μg mL^{-1}), *K.pneumoniae* (12.7 μg mL^{-1}), 6,7,8,9-Tetra hydro-5*H*-5-(2’-hydroxy phenyl)-2-(4'-fluoro benzylidine)-3-(4-nitrophenyl amino) thiazolo quinazoline 5d exhibited highest anti-fungal activity against *A.niger* (MIC: 11.2 μg mL^{-1}) and *C.albicans* (MIC: 13.9 μg mL^{-1}).The synthe-

SCHEME

Synthesis of 6,7,8,9-Tetra hydro-5*H*-5-(2'-hydroxy phenyl)-2-(4'-substitutedbenzylidine)-3-(4-nitrophenylamino)thiazoloquinazoline



sized compounds were active against all the tested micro-organisms with a range of MIC values for *S.aureus* (9.4-23.6 μg mL^{-1}), *S.epidermidis* (9.6-25.2 μg mL^{-1}), *M.luteus* (10.4-26.7 μg mL^{-1}), *B.cereus* (10.2-22.2 μg mL^{-1}), *E.coli* (11.3-24.9 μg mL^{-1}), *P.aeruginosa* (12.1-23.9 μg mL^{-1}), *K.pneumoniae* (12.7-22.9 μg mL^{-1}), *A.niger* (MIC: 11.2-27.4 μg mL^{-1}) and *C.albicans* (MIC: 13.9-26.6 μg mL^{-1}).The potent anti-microbial activity exhibited by 5a and 5b may be due to the incorporation of electron donating groups like, methoxy and dimethyl (at 4th position of the arylidine ring). The interesting results we observed that both electrons donating as well as electrons withdrawing groups was found to increase the anti-microbial properties, whereas *p*-nitro anilines unsubstituted derivatives exhibited lesser degree of activity. The compound 5a and 5b was found to possess anti-bacterial activity almost equivalent to standard drug and considerable anti-fungal activity, all the observed zone of inhibition clearly indicated in figure-4.

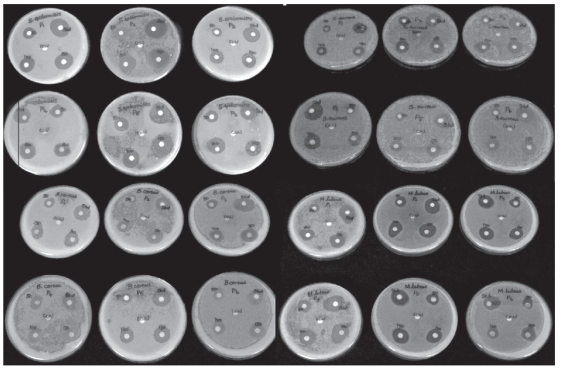


Figure 4- Zone of inhibition of the synthesized compounds **4a-d** and **5a-d**

Conclusion

In conclusion, the present study highlights the importance of phenyl hydrazine substitution at 3rd position of the thiazoloquinazoline ring features responsible for the anti-microbial property and therefore may serves as a lead molecule to obtain clinically useful novel entities in the new millennium.

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Studies to understand the effect of *Centella asiatica* on Aβ(42) aggregation *in vitro*

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Abstract

Amyloid beta (Aβ) is the major pathological and etiological factor implicated in Alzheimer's disease (AD). Aβ(42) inherently self assembles to form oligomers and fibrils through multifactorial aggregation process. The oligomers and fibrils have toxic effects and may lead to neuronal dysfunction. The inhibition of formation of oligomers and fibrils is a novel strategy in drug development programs for AD. There are limited studies to show to that *Centella asiatica* has a memory enhancing, anti-acetlylcholinesterase and antioxidant properties. But there are no studies to insight whether *C. asiatica* prevents Aβ fibrils formation from monomers and oligomers and also to understand whether *C. asiatica* destabilize the preformed fibrils. Our present study focused on, i) whether the *C. asiatica* leaf aqueous extract prevent the formation of oligomers and aggregates from monomer? (Phase I: Aβ(42) + extract co –incubation), ii) Whether the C. *asiatica* aqueous extract prevent the formation of fibrils from oligomers (Phase II- extract added after oligomers formation) and iii) whether the aqueous extract dis-aggregates the pre-formed fibrils (Phase III - aqueous extract added to matured fibrils and incubated for 8 days). The aggregation kinetics was studied using thioflavin-T assay and Transmission Electron Microscopy (TEM). The results showed that C. *asiatica* aqueous extract could not able to inhibit the Aβ aggregation both from monomer and oligomers and also could not able to dis-integrate the preformed fibrils. These intriguing results are discussed.

Key words: Aβ(42), Fibrils, Aggregation, Thioflavin-T, Transmission Electron Microscopy, *Centella asiatica*

Introduction

Amyloids are a group of misfolded proteins, implicated in the neurodegenerative disorders such as Parkinson's disease, Huntington disease, Frontotemporal dementia and Alzheimer's disease (AD) (1). The amyloid β 42 (Aβ42) is one of the amyloid protein strongly implicated in AD (2). The excessive production and accumulation of Aβ believed to be one of the major risk factor for AD (3-4). Aβ undergoes conformational change and forms deposits in the form of insoluble senile plaques in AD brain (5). The major therapeutic approaches in AD are towards the reduction of Aβ either by decreasing its production or to enhance clearance of the accumulated Aβ [6-12]. The accepted concept is that Aβ oligomers are toxic to neurons and induce cell death (4, 13-14). The therapeutic approaches in AD include acetylcholineesterase inhibitors, antioxidants, anti-inflammatory and anti-amyloidogenic agents as

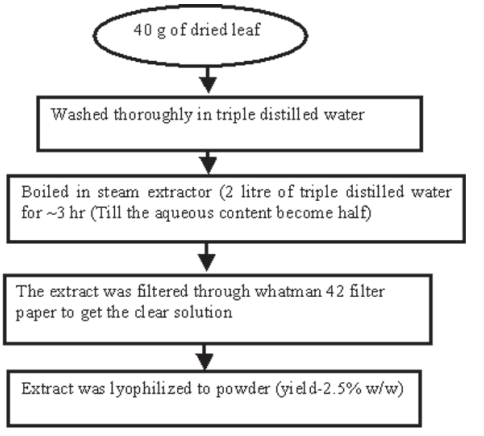
targets (15-23). The anti-amyloidogenic approach is currently active (19, 23-25). There are number of drug targets focused against amyloid load reduction and many of them have not reached the clinical trials (24-28). Now, the studies have been focused on natural products as alternative candidates for evaluating therapeutic potential against AD (21-26). *C. asiatiac* has been traditionally used in Asia to cure various ailments. The present study focused in evaluating *C. asiatica* for its anti- Aβ aggregation properties. The dried leaves of Indian penny wort (botanical name: *Centella asiatica*) is mixed with milk and consume as memory improving (29-30) and this is practiced traditionally in selected regions in India (31). There are studies on diverse effects of C. *asiatica* such as acetylcholine-esterase inhibition, antioxidant, neuroprotection, and amyloid load reduction (16, 18, 32-34). However, there are no mechanistic studies to understand whether *C. asiatica* prevents Aβ aggregation. In the current study, we have used Aβ(42), which is the most amyloidogenic peptide, for the formation of oligomers, protofibrils and fibrils. And also, we planned to map whether *C. asiatica* inhibits Aβ aggregation. We used aqueous extract of C. *asiatica* as it is traditionally used by local population in Western Ghats as brain tonic.

Materials and methods

Aβ(42) was purchased from EZ Biolabs, USA. Tris buffer, glycine, sodium hydroxide, Hydrochloric acid were from SRL, India. Thioflavin –T was procured from ICN Biomedicals Pvt. Ltd, USA and Copper grids (200 mesh size) were brought from Sigma chemicals, USA and uranyl acetate was procured from BDH Laboratory chemicals Division, India.

The C asiatica was procured from local vegatable market, Mysore and it was identified by authenticated botanist (Taxonomic deposit number is 9831).

Flow chart for the preparation of aqueous-leaf extract of *C. asiatica*



The 40 g dried leaves of *C. asiatica* was washed thoroughly in triple distilled water for four times. Two litres of triple distilled water was added to steam extractor, boiled till it become half volume (one liter). The one litre aqueous extract was filtered using Whatman 42 filter paper to get clear solution. The clear solution of the extract was lyophilized to get dry powder. The yield of the extract was 2.5% (w/w).

To evaluate the anti-amyloidogenic property of aqueous extract of *C. asiatica*, the following experiments were designed *in vitro* using the three-phase study protocol. Phase I: To understand the prevention of Aβ aggregation from monomer. Phase I reaction mixture was as follows: 100μM of Aβ was incubated with 100μg of lyophilized aqueous extracts *of C. asiatica* in a total reaction mixture of 300μl containing 10mM Tris-Cl (pH 7.4) at 0 hrs at 37 ^{0}C. Aliquots of 20μl (10 μM) were drawn each time from incubated sample at intervals of 0, 6, 20, 72 and 96 hrs. Phase II: To understand the prevention of formation of aggregates from oligomers. Freshly prepared Aβ was allowed to form oligomers till 20 hrs following the

protocol with slight modification [35] and then 100µg of lyophilized extract was added. The aggregation kinetics was studied to follow the formation of aggregates from oligomers as function of time (20-96 hrs) and aliquots were taken at 20, 36, 48, 72 and 96 hrs for thioflavin-T and TEM study. Phase III: To understand the efficacy of extract to dis-integrate the pre-formed fibrils. Freshly prepared Aβ was allowed to form matured fibrils by 96 hrs and then 100µg of lyophilized extract was added and followed the dis-integration of fibrils at 8 days.

Thioflavin-T assay

The thioflavin-T assay was followed to study Aβ aggregation kinetics. Thioflavin-T specifically binds to aggregates but not to soluble monomers. 25µl of (1mM) thioflavin-T was added to 1000µl of total reaction volume containing 5µM Aβ. The thioflavin-T fluorescence was measured at an excitation and emission wavelengths of 446nm and 482nm, respectively using a F4500 Hitachi Fluorescence Spectrometer. The background thioflavin-T fluorescence intensity was subtracted from the experimental values. The thioflavin-T fluorescence data was analyzed for standard error using origin 6.0.

Transmission Electron microscopy (TEM) study

Transmission Electron Microscopy (TEM) study was conducted to detect presence or absence of aggregates. 10µL of incubated sample was placed on carbon coated copper grid (200 mesh size) and allowed for one min and excess sample was wicked off with lens paper and then negatively stained by transferring the grid face down to a droplet of (2% (w/v) uranyl acetate for one min before wicking off the solution. Then the grids were air dried for an hour. Four individual experiments were carried out for each sample. The grids were completely dried to avoid moisture and then scanned under JOEL 1010 TEM.

Results

The effect of *C. asiatica* on the Aβ aggregation is analyzed as follows.

Phase I: Inhibition of the formation of aggregates from monomers: Fig 1A shows three phases of aggregation kinetics of Aβ as monitored by thioflavin-T fluorescence as a function of time (0-96 hrs). The aggregation kinetics followed a sigmoidal curve. The thioflavin –T data indicated a lag period upto 20 hrs, where thioflavin-T fluorescence intensity is static indicating the presence of monomers only. After 20 hrs, there is an intermediate phase from where oligomers and other intermediate forms form till 48 hrs. The thioflavin –T fluorescence steeply increases in this phase indicating formation of misfolded intermediates. The other phase is the saturated phase where fully matured fibrils are formed. This phase is from 48 to 96 hrs. The thioflavin –T fluorescence is higher and static in nature indicating matured fibril formation. In the presence of the aqueous extract, the sigmoidal pattern of Aβ aggregation kinetics is followed a similar pattern with no significant reduction in fluorescence intensity. This indicates that *C. asiatica* could not be able to prevent the Aβ aggregation from monomers. Fig 1B shows the thioflavin-T fluorescence at 0, 6, 20, 72 and 96 hrs time intervals. For Aβ alone, as the time increases, the thioflavin-T fluorescence increases. And in the presence of aqueous extract, thioflavin-T fluorescence do not significantly altered; supporting the concept that extract could not able to prevent Aβ aggregation. The samples represented in Fig 1B are taken for TEM study. Fig 1C shows results of TEM study for the presence or absence of aggregates at different time intervals (0, 6, 20,72 and 96 hrs). The TEM data clearly shows that when Aβ alone is allowed to aggregate from 0 to

96 hrs, the formation of fibrils is in time dependent aggregation kinetics. There are no aggregates from 0 to 20 hrs. The aggregates start growing from 72 and 96 hrs (Fig 1C). Even in the presence of *C. asiatica*, both at 72 and 96 hrs the aggregates are seen indicating that the extract could not able to totally prevent the formation of fibrils (Fig 1C). Both thioflavin-T and TEM data clearly supports that the extract could not able to totally prevent the formation of aggregates.

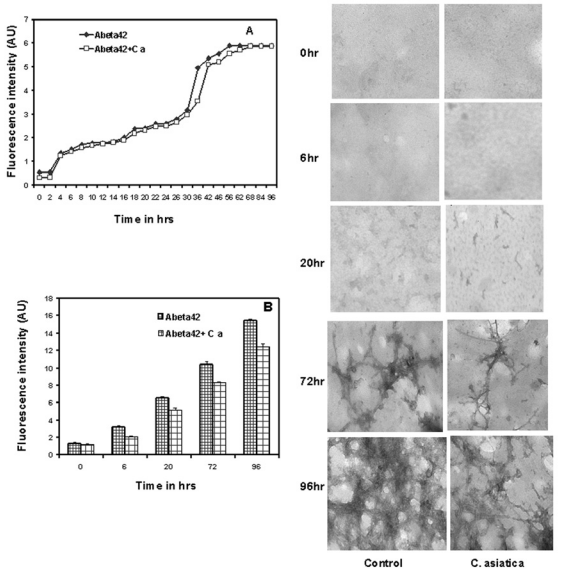


Fig 1: Phase I (monomer to aggregates): A- The reaction mixture containing 50μM Aβ(42) co-incubated in absence or presence of 100 μ g of extract at 37°C in Tris-Cl buffer (pH 7.4). Aliquots are taken at 2 hrs intervals from 0-96 hrs. The fluorescence of thioflavin – T is recorded with excitation and emission wavelength of 446nm and 482nm respectively. B- Bar diagram showing the thioflavin-T fluorescence of Aβ(42) in presence or absence of *C. asiatica leaf* extract at 0, 6, 20, 72 and 96 hrs. C- Transmission Electron Microscopic study: The reaction mixture contain 50μM Aβ(42) co-incubated in absence or presence of 100 μ g of extract at 37°C in Tris-Cl buffer (pH 7.4). The aliquots are taken at 0, 6, 20, 72 and 96 hrs and analyzed for presence or absence of aggregates.

Phase II: Inhibition of the formation of aggregates from oligomers: Fig 2A shows the

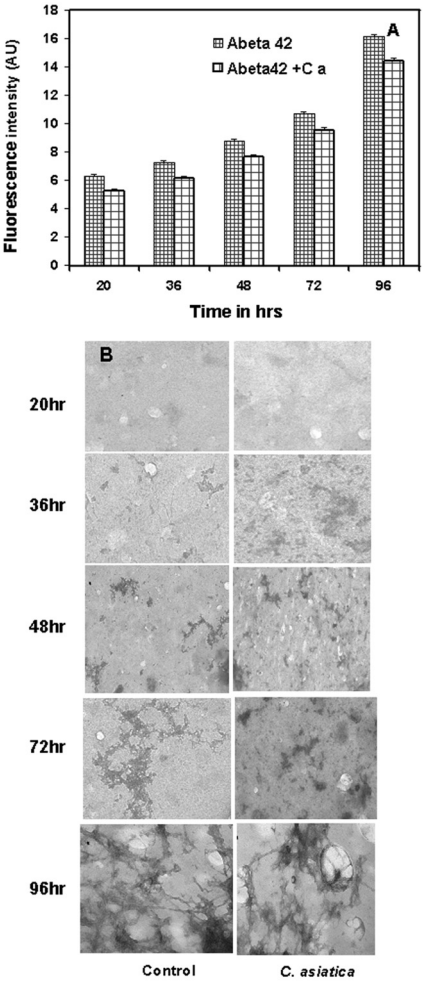


Fig 2: phase II (Oligomers to aggregates): A- Bar diagram showing the thioflavin-T fluorescence of Aβ(42) as a function of time: Aβ(42) is allowed to form oligomers for 20 hrs and *C. asiatica leaf* extract is added at 20 hrs of incubation. Aliquots are taken at 20, 36, 48, 72 and 96 hrs and thioflavin-T fluorescence emission was measured at 482nm. B-Transmission Electron Microscopy study: Aβ(42) is incubated for 20 hrs and *C. asiatica leaf* extract is added at 20 hrs of Aβ(42) incubation. Aliquots are taken at 20, 36, 48, 72 and 96 hrs. Aliquots are analyzed for the presence or absence aggregates using electron microscopy.

results of phase II. Thioflavin-T fluorescence data of Aβ is analyzed with and without extract at different time intervals (20, 36, 48, 72 and 96 hrs). Aβ is allowed to form oligomers for 20 hrs, and at 20 hrs, extract is added and the Aβ aggregation process is monitored at different time intervals. The thioflavin-T fluorescence has increased from 20 to 96 hrs indicating the formation of fibrils from oligomers stage. In the presence of *C. asiatica*, the thioflavin-T fluorescence values do not decrease significantly indicating that the extract could not inhibit the formation of fibrils from oligomers. Fig 2B shows the results of TEM of phase II. The formation of fibrils increased with time from 20 to 96 hrs in Aβ alone and in the presence of *C. asiatica,* also the fibrils are seen but less in number. This indicates that *C. asiatica* could not be able to totally prevent fibril formation from oligomers (Fig 2B).

Phase III: Dis-integration of pre-formed fibrils: Fig 3A shows the results of thioflavin-T fluorescence assay of phase III. The matured fibrils after 96 hrs are allowed to further grow till 8 days. The fluorescence has increased from 96 to 8 days in Aβ alone and in the presence of *C. asiatica* extract no significant reduction in thioflavin-T fluorescence was observed. This indicates that extract did not dis-integrate the preformed fibrils even after 8 days of incubation. Fig 3B shows the results of TEM of phase III. At 96 hrs, there are matured fibrils with extensive branching in sample having Aβ alone. The extract is added to 96 hrs-matured fibrils and then incubated for 8 days. The extract could not disintegrate totally the pre-formed fibrils even after 8 days of incubation.

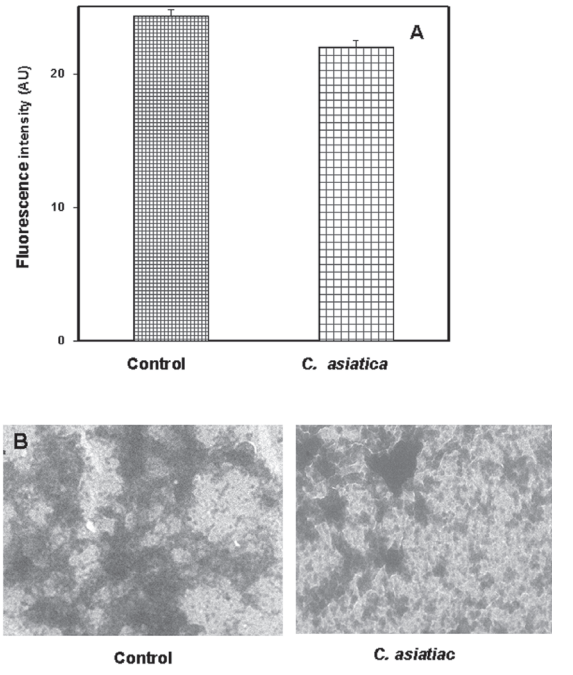


Fig 3: Phase III (Disintegration of preformed fibrils): A- Bar diagram showing the thioflavin-T fluorescence of Aβ(42): Aβ(42) is incubated for 96 hrs and *C. asiatica leaf* extract is added and further allowed to aggregate for 8 days. Thioflavin-T fluorescence measured at emission at 482nm. B- Transmission Electron Microscopy study: Aβ(42) is incubated for 96 hrs and then *C. asiatica leaf* extract is introduced and allowed to aggregate for 8 days. The aliquots are analyzed for the presence or absence of aggregates in electron microscopy.

Discussion

Alzheimer's disease is a progressive neurodegenerative disease affecting millions of people worldwide. The etiological factors include oxidative stress, inflammation, Aβ over expression, elevation in metals etc. (2, 36-37). Among the risk factors implicated, Aβ is strongly associated with AD [3, 38]. The therapeutic approaches in AD include, reducing the amyloid production or enhancing the clearance of amyloid load in AD [39-40]. The ancient Indian system of medicine, Ayurveda has described traditional use of herbal medicinal therapies for the treatment of dementia (29). In particular, *C. asiatica* has been

listed in ancient Indian Ayurveda medical text Caraka Susmita as a treatment for dementia. The leaves of *C. asiatica* is used as a memory booster in some regions of India [30-31]. The animal studies have shown that extracts of *C. asiatica* improves memory in rats (32-34). The *C. asiatica* also found to improve memory, behavior and performance tests of mentally retarded children (41). Kumar and Gupta (42) have shown that *C. asiatica* prevents streptozotocin induced cognitive deficits in rats. Subathra et al. (43) reported that *C. asiatica* reduced the protein carbonyls in the aged rat brain. Further, Kumar et al. (34) have demonstrated that *C. asiatica* significantly decreases the acetylcholine esterase activity in colchicine induced cognitive impairment and oxidative stress. Nalini et al. (44) showed that *C. asiatica* showed improvement in the avoidance task in rats. Rao et al. (45) reported that *C. asiatica* able to improve the brain function of mice if treated during postnatal period. Recently, Dhanasekaran et al. (16) have reported that *C. asiatica* moderately decreased Aβ(40) and Aβ(42) load both in cortex and hippocampus region of PSAP Alzheimer's disease mice model. But the reduction of Aβ(40) and Aβ(42) did not improve the Y-maze and open field behavior tests. The mechanism of reduction in the amyloid load is not clearly understood. There are no studies to show the effects of *C. asiatica* on Aβ aggregation kinetics. Our results showed that C. *asiatica* aqueous extract could not significantly inhibit the Aβ aggregation either from monomer and oligomers and also not be able to dis-integrate the preformed fibrils. Based on our data and literature findings, we propose the following mechanism of action. Aβ exists in monomer form and monomers will be in random coil conformation. Aβ in suitable condition self- aggregates into fibrils. The aggregation process will pass through different conformation in the following order: random coil, misfold , β- sheet/ β- turn or in combination of all these conformations. The fully aggregated long fibrils will be either in β- sheet/ β- turn conformation. The aqueous extract of *C. asiatica* could not stabilize random coil of monomers hence could not prevent fibril formation. However, we further propose that *C. asiatica* extract may be acting by other possible pathways such as (i) enhancing the α- secretase pathway of APP processing or inhibiting β- secretase activity; (ii) may be acting as anti-oxidant, so that oxidative stress will be reduced. And Oxidative stress enhances Aβ expression; (iii) may enhance clearance mechanism of accumulated amyloid; or (iv) may be acting as anti-inflammatory candidate. All these events together may be helpful for *C. asiatica* as brain tonic or memory or cognitive function enhancer. Further work is needed to understand more on the efficacy of *C. asiatica* as a therapeutic intervention molecule.

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Prof. Dr. Gopi Krishna Podila (1957-2010), an internationally renowned Indian American scientist dedicated to molecular research on trees and plant-fungal interactions - Obituary

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The sad sudden demise of Dr. Gopi K. Podila on February 12, 2010, an eminent academician at University of Alabama in Huntsville (UAH), was a great shock to the scientific world, particularly for Plant Microbial Scientists. Dr. Podila was born on September 14, 1957 in India and had his education in biology, plant pathology and soil microbiology up to Masters level carried out at Guntur, Andhra Pradesh, India with a commitment and a zeal for higher learning.

Dr. Podila concentrated very successfully in his scientific career not solely but majorly on interactions between fungi and plants. Another Masters degree was completed in the USA at the Louisiana State University in the field of plant pathology and then in 1987 his PhD at the Indiana State University in molecular biology. This was done at the early days of molecular biology, at times where molecular methods were by far not as advanced and where most molecular biologist had to act as pioneers in establishing new methods and in more and more optimising and adapting them to newly arising situations. Accordingly, Dr. Podila was amongst the first who studied viruses in plant pathogenic fungi (1-4) and new methods of in vitro translation were readily transferred from viral proteins to mushroom enzymes (5).

Subsequently, Dr. Podila made a move as a Post-Doc at Ohio State University to the well known laboratory of Prof. Dr. Papachaan Kolattukudy that was very famous at the late 1980s for the innovative cutinase work on fungal plant pathogens. Dr. Podila participated with major scientific contributions in the success story of establishing the molecular regulation of fungal cutinase genes and the biochemical actions of the enzymes in early steps of fungal infection and the development of diseases (6-12). Noticeable from this time are his first publications in the highest ranking scientific journals Science (6), Nature (7), and Proceedings of the National Academy of Sciences, USA (8). Being a brilliant and reflective scientist, he linked his understanding obtained by his former virus research to the new knowledge on cutinases: cutinase gene-expression was shown to be suppressed in fungal strains with virus RNA (13) and this lead to first ideas in biotechnology of fungal disease research (14).

In the next step of his fast developing career, being appointed first as an assistant professor and then an adjunct associate professor at Michigan Technological University (MTU) in the School of Forestry, trees moved into the focus of Dr. Podila´s attention. Various methods in plant molecular biology were developed (15-17), plant and microbial genes cloned and their encoded products studied (18-21) and, most importantly and again very innovatively, transgenic trees were produced and characterized (22-27). Specifically, lignin metabolism was studied and modified in trees by genetic engineering. Trees modified in such way in lignin composition may have better value in industrial applications, e.g. in paper production (27,28).

In addition at MTU, important ecological questions were addressed such as the harmful effects

and gene responses of raising ozone concentrations in aspen and how these interlink with CO_2 (29-33). This type of research Dr. Podila continued also after he moved in 2002 to his last appointment to act as a Professor and Chair at the Department of Biological Sciences of the UAH (34-36) and further genes from trees were cloned and characterized (36-39) and protocols for poplar transformation optimized (40).

At Huntsville changing to the "good" fungi, in more recent years Dr. Podila concentrated at symbiotic interactions studying fungi forming ectomycorrhizae with trees. Again, new urgently needed molecular tools were developed such as fungal transformation (41,42) and, now, many interesting fungal genes were cloned and studied in their functions in metabolism, development and interaction with the tree hosts, mostly genes from symbiotic species (43-51) but occasionally also from a pathogenic fungus (52). Much pioneering work was done particularly on the basidiomycete *Laccaria bicolor* (42-49,53-55) forming ectomycorrhizae with a broad range of trees (56). Therefore not surprisingly, Dr. Podila had a leading part in the first international genome sequencing project of an ectomycorrhizal fungus (56-58). As another highlight of Dr. Podila´s exceptional scientific career, the genome of *L. bicolor* was published in 2008 in Nature (58). In the advent of large genome sequencing projects, mass analysis of genes by transcriptomics and proteomics has been made possible and Dr. Podila used such modern molecular techniques to get deeper insight in fungal-plant interactions (55,56,59) and in comparing life-styles of plant-pathogenic and symbiotic fungi (60,61).

Dr. Podila´s central, internationally well recognized position in mycorrhizae research was honoured by his nomination of Councilor of the International Symbiosis Society. He served as member of editorial boards for the renowned international science journals Journal of Plant Interactions, New Phytologist, Physiology and Molecular Biology of Plants and Symbiosis and he acted as editor of various scientific books (62-65).

Dr. Podila was known to be as one of the motivating teachers at UAH for about 8 years and earlier at MTU and he played an important role in designing course work and projects in various modern areas.

Regardless of whether we have personally known Dr. Podila as friend and colleague for decades (KRSSR) or only in later years of his scientific life (UK), we mourn the loss of a very kind, modest and special person that in his quiet understanding way ever so often has been helpful to us and so many others. We mourn the loss of a teacher who had so much to give to students and colleagues and the loss a dedicated, highly knowledgeable and innovative scientist that drove research to highest altitudes and new fields of study. We feel with Dr. Podila´s wife, daughters and other family and offer our deepest sympathy to them.

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NEWS

Prime Minister Inaugurates 97th Indian Science Congress

January 3, 2010 Thiruvananthapuram, Kerala: Prime Minister, Dr. Manmohan Singh said that the strong developments in Indian Science and Technology has led the way to re-emerge in education, human resource and as a knowledge power in the 21st Century. In the past few years Indian Government has invested heavily in expanding and upgrading the science, technology and innovation system in supporting a more broad-based educational system in our country and determined to ensure that what we have announced does get implemented. The country is facing new challenges of climate change and the management of our scarce water resources, food security and disease control and our success will depend critically on the quality of our institutions of science and technology.

Science and Technology Policy of Japan

Prime Minister of Japan Yukio Hatoyama detained the 88th meeting of the Council for Science and Technology Policy at the Prime Minister's Office. During the meeting, discussions took place on basic policy for the management of the support program for cutting-edge next-generation research and development, and efforts to realize a prioritized and efficient budget related to science and technology. In his address, Prime Minister Hatoyama said, "I call upon you to take the lead in science and technology policy, so that young researchers and female researchers in particular can demonstrate their abilities to the fullest extent under the growth strategy that we formulated at the end of last year."

Union HRD Minister Kapil Sibal at tough laws for foreign varsities

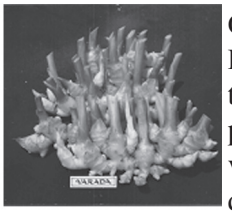
Union HRD Minister, Kapil Sibal wants to formulate tougher laws before allowing foreign universities to set up shop in India. Speaking to media persons during a visit to the Indian School of Business (ISB), Hyderabad, Sibal said that the Central Government did not want any fly-by-night institutions to take advantage of its invitation. "Foreign universities cannot transfer the profits earned in India to their motherlands but should invest back in Indian campuses." Sibal said that there were unnecessary apprehensions about foreign universities entering the Indian arena. "Why should we not have world-class institutions like ISB here? Why should our children go abroad for quality education and pay high fee when we can have the same quality of education here at one-third of the fee collected by foreign universities?" He wanted to know and felt that domestic universities need not be afraid of competition from foreign universities as it will only help us to improve educational standards in the country. The Union minister appreciated the management of Indian School of Business (ISB) which entered into a memorandum of understanding with the MIT Sloan School of Management to develop two new institutes.

Indian Prime Minister unveils 'Solar Valley' vision

January 11, 2010: Prime minister, Manmohan Singh launched India's solar energy mission under the national action plan on climate change. The country aims to generate 20,000 megawatts (MW) of electricity from the sun by 2020. "If the ambitious mission is to become a living reality,

we will have to create many 'solar valleys' on the lines of the silicon valleys that are spurring our information technology industry across the four corners of our country," said Singh. These valleys will become hubs for solar science, solar engineering and solar research, fabrication and manufacturing. "Singh said technological innovation is the key to the mission's success. "We will need to find ways of reducing the space intensity of current solar applications including through the use of nanotechnology with cost-effective and convenient storage of solar energy beyond daylight hours to explore hybrid solutions, combining solar power generation with gas, biomass or even coal-based power.

A Ginger For Candy-Varada



Ginger (*Zingiber officinale* Rosc.) is one of the important spices with aroma and pungency with many ginger varieties in India and abroad differing in their qualities and yield. Dry gingers are world class ones, value added products like ginger flakes, ginger beer, ginger wine, ginger cookies, ginger paste, salted ginger, ginger candy etc are made from ginger.

'Varada' variety of ginger developed by the Indian Institute of Spices Research, Kozhikode, Kerala is not only the first ever ginger variety evolved in the state but also is found most suitable for ginger candy. Trade Craft, a UK based charitable fair trade organization in collaboration with the Avoly (Moovattupuzha) based Nadukkara AgroProcessing Co.Ltd (NAPL) is now making Varada ginger candy in a very large scale!

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TWEAKing on muscle wastage

Skeletal muscle wasting is the major reason for morbidity and mortality in several chronic disease states including cancer, AIDS, chronic heart failure, sepsis, diabetes, chronic obstructive pulmonary diseases, and chronic renal failure. Skeletal muscle wasting also occurs in the conditions of disuse such as nerve injury, bed rest, immobilization, and unloading. Proinflammatory cytokines are considered as the key mediators of muscle-wasting in many chronic conditions. However, the involvement of cytokines in skeletal muscle loss in disuse condition has never been documented.

"Muscle wasting is not only a concern related to many chronic conditions, but it is a serious problem afflicting astronauts who spend any length of time in space and often require physical rehabilitation upon return." TWEAK induced muscular atrophy has the potential of generating novel therapeutics for muscular atrophy related to the interaction between TWEAK and a receptor called Fn14 is very important because it has implications for potential drug therapies that might use this interaction as a target." Future studies will help to determine the full range of clinically relevant settings in which the TWEAK/Fn14 pathway is operative, and in which inhibition of this pathway is sufficient to preserve functional muscle." Muscle atrophy as a complication of illness can hasten death by affecting a patient's ability to fight infection and in some cases rendering patients unable to eat or breathe on their own.

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Menstrual Blood-A good source of Stem Cells

The menstrual blood contains a viable source of stem cells to be utilized or preserved for future use of vast range of therapies and stem cell research. Menstrual blood contains millions of stem cells that demonstrate properties similar to bone marrow and embryonic stem cells. The stem cells found in menstrual blood rapidly multiply, turning into possibly every cell type in the human body. The stem cells from the intact endometrium appear to be mesenchymal stem cells (MSCs). Menstrual Stem Cells (MenSCs) are distinctly different not only in their undifferentiated state, but they can differentiate as well. MenSCs though morphologically appearing as mesenchymal, were found to express only some, but not all, proteins characteristic of MSCs. Additionally, MenSCs were reported to be able to differentiate into, or become, cells from the three different germ layers: mesoderm (muscle, bone, fat, cartilage, and endothelial cells), ectoderm (neurons) and endoderm (liver, pancreas, and lung cells). The new stem cells from the menstrual blood also grow readily, which is an important advantage. Due to their ease of collection and isolation, MenSCs would be a great source of multipotent cells as they exhibit this property along with their ability to differentiate. The MenSCs expanded rapidly and maintained greater than 50 percent of their telomerase activity when compared to human embryonic stem cells and better than bone marrow-derived stem cells. These cells may be used in the treatment of a number of serious diseases, such as osteoporosis, stroke, Alzheimer's and Parkinson's disease. The cells may even one day be used for customized anti-aging or sports medicine treatments. The ideal cell would also have the ability to be used in an allogenic manner from donors for optimal immunogenic compatibility.

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Key difference between reprogrammed adult mouse cells and embryonic stem cells discovered.

Mouse stem cells can be used to create neurons (above) for the screening of drugs. Stem-cell researchers have puzzled over why reprogrammed cells taken from adult tissues are often slower to divide and much less robust than their embryo-derived counterparts. Now, a team has discovered the key genetic difference between embryonic and adult-derived stem cells in mice. If confirmed in humans, the finding could help clinicians to select only the heartiest stem cells for therapeutic applications and disease modelling. Induced pluripotent stem (iPS) cells are created by reprogramming adult cells, and outwardly seem indistinguishable from embryonic stem (ES) cells. Both cell types are pluripotent they can form any tissue in the body. Yet subtle distinctions abound. Last month, for example, Su-Chun Zhang and his colleagues at the University of Wisconsin–Madison compared the ability of both types of pluripotent cell to form human neurons in a laboratory setting, and found that iPS cells did so with markedly lower efficiency than ES cells (B.-Y. Hu et al. Proc. Natl Acad. Sci. USA 107, 4335–4340; 2010). Published online 31 March 2010 | Nature 464, 663 (2010)

Elie Dolgin

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Testing time for stem cells: Industry turns to cell lines for drug screens

A deal between Roche and two academic institutions will focus on stem-cell-based drug screening. Roche: The drug industry is keener on stem-cell technologies than ever before and not just as a source of new treatments. A wave of new partnerships aims to use stem cells as a way to screen other potential drug candidates. In the latest such example, Roche last week announced a deal worth some US$20 million with Harvard Univer-

sity in Cambridge, Massachusetts, and Massachusetts General Hospital in Boston. Roche, based in Basel, Switzerland, will use cell lines and protocols developed by academic researchers to screen for drugs to treat cardiovascular disease and other conditions.

Because relevant human cell types are often unavailable, current screens tend to use cells from rodents or human tissues other than the ones researchers want to target and that stem cells could provide exactly the type of cells relevant for an assay. The deal is the latest in a string of similar partnerships. Within the past 15 months, Pfizer of New York and GE Healthcare of Chalfont St Giles, UK, signed deals geared towards using stem cells in drug discovery with the California companies Novocell of San Diego and Geron of Menlo Park, respectively. "What needs to be demonstrated is the actual application of the technology," adds John Walker, the chief executive of iPierian, a stem-cell company in San Francisco, California. The firm has created motor neurons using induced pluripotent stem (iPS) cells derived from people with and without spinal muscular atrophy, a neurodegenerative disease. Eventually, this work could lead to what Walker calls an "in vitro clinical trial" in which iPS cells derived from a wide variety of individuals could be used to predict patients' response to a drug. Published online 9 February 2010 | Nature 463, 719 (2010).

Monya Baker

THAPAR UNIVERSITY

National Conference

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Fourth Annual Convention of Association of Biotechnology and Pharmacy

Fourth Annual Convention of Association of Biotechnology and Pharmacy and the National Conference on "Emerging Trends in Biopharmaceuticals – Relevance to Environment and Health" is being organized jointly by Department of Biotechnology and Association of Biotechnology and Pharmacy at Thapar University, Patiala during 11-13 November, 2010.

For further details contact

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