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Development and Validation of Reversed Phase HPLC Method for Analysis of Immunotherapeutic Peptide PADRE and its Applications

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Abstract

A reversed phase high performance chromatography (RP-HPLC)method was developed and validated for the quantification of PADRE, a peptide with proven immunogenic activity *in vitro*.The developed method was used to determine PADRE stability within biomatrices like plasma, intact tumor and tumor homogenate *in vitro* and to determine PADRE release from its PLGA base dmicroparticle formulation.A reversed phase C8 column was used and the elution was performed using a concave gradient flow. Mobile phase consisted of acetonitirile and 0.01M phosphate buffer at 10:90 ratio containing 0.01% trifluoroacetic acid, adjusted to pH 3, was increased using a concave gradient to 40:60 v/v in 15 minutes. The retention time of PADRE was found to be 10.4 minutes. The method was linear in the range of 0.5-100 µg/ml and the regression coefficient was obtained as 0.9997. The method was found to be accurate with recovery in the range of 97.80 and 104.64%. Precision ofthe proposed method was established by determination of intra day and inter day variability using standard solutions. The percentage relative standard deviation values were all within acceptable range. The detection and quantitation limit for the method were found to be 0.156µg/ml and 0.5 µg/ml. Utilizing the proposed method, degradation profiles of PADRE within biomatrices were obtained. PADRE exhibited rapid and maximum degradation within plasma incomparison to intact tumor or tumor homogenates. PADRE was encapsulated within PLGA microparticles as a means to improve the stability *in vivo*. The proposed HPLC method was also used to characterize PADRE encapsulation within its PLGA based microparticle dosage form. The encapsulation efficiency was found to be 27.2±6.3% and the surface associated peptide was quantified as 21.6±2.2%. The release of PADRE from the dosage form was found to be biphasic. The method may further be utilized to quantify PADRE at tumor site and in plasma upon systemic injection and also to study improvement in PADRE stability using enzyme inhibitors and various drug delivery systems.

Keywords: PADRE, Reversed phase HPLC, Stability, Release

Introduction

The utility of peptide-based vaccines has been exploited for treatment of various types of cancers. Certain tumor associated antigens (TAAs) expressed by cancer cells may be utilized as a trigger to generate a potent systemic immune

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response to specifically destroy proliferating and metastasizing cancer cells (1). The TAAs bind to the peptide sequences present onmajor histocompatibility complex (MHC), also called HLA, which essentially are proteins expressed on most cell surfaces.This binding is effective only when certain amino acidsare preserved in thepeptide sequence at fixed positions. The TAA-MHC complex is then recognized byT lymphocytes to generate an immune response. The ability of a peptide to generate an immune response therefore depends on how efficiently it binds to MHC and also on the efficiency of recognition of the TAA-MHC complex by the T cells (2).

Several peptides have shown activity when tested on *in vitro* culture systems and also produce effective immunogenic responses *in vivo* when coated onto surfaces of tumor cells before inoculation in animals. However potent immunogenic responses are not observed when the peptide is injected systemically. The possible reasons for this could be the physical, physiological and metabolic barriers presented by tumor tissue. It is important to determine if the peptide is susceptible to enzymatic degradation byexo and endo peptidases present within biological matrices like plasma, extracellular matrix and tumor resulting into loss of immunogenic activity (3).

Pan DR Reactive Epitope (PADRE) is a syntheticnon-naturallinear peptidebelonging to the MHC II family. The peptide PADRE having a 13 amino acid sequence, aK(X) VAAWTLKAAa, has a high binding affinity towards a number of MHC allelic variants which is advantageous in imparting immunogenicity. Also, when tested on *in vitro* culture systems using T cell proliferation assay, PADRE has shown to be a powerful antigen with 100 fold higher potency than a control tetanus toxoid (4,5). With its proven immunogenicity *in vitro*, the peptide has a strong potential as a vaccine for cancer therapy.However when injected systemically, the peptide failed to produce delayed type hypersensitivity or tumor rejection, possibly due to physical and metabolic barriers (6), which may be overcome by suitable delivery strategies.

The development of a peptide as a therapeutic demands a reliable and rapid analytical method. Previously RP-HPLC has been used for the analysis of immunogenic peptides such as a 34 amino acid peptide called HEL (Hen egg white lysozyme) has been characterized by HPLC and separated from degradation products upon trypsin and chymotrypsin treatment.The method however was not validated to test the sensitivity, accuracy and precision and had along run time of 60 minutes (7). The present work aimed at development and validation of a reversed phase HPLC method for determination of PADRE with good sensitivity and short analysis time. The method will be particularly useful to determine the levels of PADRE at the tumor site. It will alsobe useful to determine stability of PADRE within biological matrices like plasma, solid tumor and tumor homogenates and to characterize the encapsulation of PADRE in drug delivery systems like microparticles (8,9) and to study subsequent release kinetics of PADRE from these systems.

Materials and Methods

The peptide PADRE was synthesized by Genemed Synthesis Inc., San Francisco, USA. All reagents and solvents, namely,trifluoroacteic acid (reagent grade), monobasic potassium phosphate (reagent grade) and acetonitrile (HPLC grade) were obtained from Sigma Aldrich, St.Louis, USA. Buffers were prepared in deionized water.

***Instrumentation*:** The high performance liquid chromatography was performed using LC- HP 1100 series (Hewlett Packard Corporation, USA) system, equipped with G1311A pump, G1313A auto sampler and G1315A diode array detector. The system was equipped with a NovapakC8 reversed phase column (Waters corporation, USA), 5 μm particle size, 4.6 mm internal diameter and 150 mm in length.The data analysis and acquisition was executed using Chem Station software (Agilent, Santa Clara, CA, USA).

***Optimization of Chromatographic conditions*:** The separation was performed using a concave gradient flow. At the start of the separation, the mobile phase consisted of acetonitrile (10%) and 0.01 M phosphate buffer containing 0.01% trifluoroacetic acid, adjusted to pH 3, (90%) at a flow rate of 1 ml/min. The mobile phase ratio was increased from 10:90% v/v to 40:60%v/v from 0 to 15 minutes using a concave gradient.A wavelength of detection of 210 nm was used. For each study, mobile phase was prepared by filtration through 0.45 μm nylon filter and was degassed prior to use.

***Preparation of standard solutions*:** PADRE was solubilized in phosphate buffered saline (PBS). Standard solutions were prepared at the concentrations 0.5, 1, 2.5, 5, 20, 25, 50, 75 and 100 μg/ml. Freshly prepared stock solutions were used to perform all analysis and final dilutions of standard solutions were prepared in mobile phase.

***Validation of the method*:** The developed HPLC method was validated using certain analysis parameters like linearity, accuracy, precision, range, limit of detection and limit of quantification (9,10). Various concentrations of the standard solution of PADRE were run and the peak area was determined by integration. A standard curve of concentration (μg/ml) v/s peak area (mAU) was plotted and regression coefficient of the plot was determined as a measure of linearity.Accuracy of the method was assessed by testing different concentrations of standard solution of PADRE and determining the percentage recoveryat each concentration (n=6). The intra-day and inter-day variability was studied to determine precision of the method. To obtain the intra-day and inter-day variation, each standard solution of PADRE was analysed six times on five different days. The percentage of relative standard deviation (% RSD), also known as % CV was determined as a measure of precision. The limit of quantification (LOQ) and limit of detection (LOD) were determined as per the method specified in ICH guidelines using empirical method (11, 12).

Application of the method

a) The developed HPLC method was utilized for determining the stability of PADRE in biomatrices like mouse plasma, intacttumor and tumor homogenate.Fresh plasma and tumor were extracted from tumor bearing mice after CO_2 euthanasia. For preparing tumor homogenate, the tumor is homogenized in 2 ml of phosphate buffer and centrifuged to obtain a peptidase rich supernatant, which is used for further studies.The peptide was incubated in PBS, mouse plasma, solid tumor and homogenized tumor (supernatant)at 37°C and aliquots were withdrawn at 0, 1, 2, 3, 18 hours. The plasma samples were de-proteinated with equal volume of acetonitrile and centrifuged at 10,000 rpm for 10 minutes and the supernatant was subjected to further analysis. All the samples were analyzed by the proposed HPLC method.

b) The proposed method was also used to determine the encapsulation efficiency of the peptide PADRE within poly lactic co glycolic acid (PLGA, M.W. 60,000) microparticles. A w/o/o emulsion method was used for preparation of the microparticles. Briefly, the PADRE was dissolved in 0.5% sodium lauryl sulfate and then

dispered in acetonitrile:dichloromethane (50:50) as the oil phase. The primary emulsion so formed was homogenized in mineral oil and double emulsion so formed was evaporated under vaccum to remove the organic phase. The resultant particles were hardened using hexane. For determination of encapsulation efficiency (%EE), the microparticles were digested in acetonitrile and peptide was extracted in water and analyzed using the proposed HPLC method.The amount of surface associated PADRE was determined by simply washing the microparticles with water. The release of PADRE from microparticleswas studied at 0, 2,4, 6, 8 and 24 hours in PBS using the HPLC method.

Results and Discussion

***Optimization of method*:** A method for determination of a novel peptide PADRE by reversed phase high performance liquid chromatography was proposed. The development of the HPLC method for PADRE involved optimization of the mobile phase to obtain a relatively sharp and symmetrical peak with a suitable retention time. Using the proposed HPLC method, PADRE showed a retention time of 10.4 minutes. Fig. 1 is a representative chromatogram for the proposed method for determination of PADRE. Pure synthetic grade PADRE was used in the development of this method. No additional peaks due to any possible degradation of the peptide were observed in the chromatogram, indicating that the method conditions and solvents were suitable for the analysis.

***Linearity*:** The standard curve was obtained by plotting the peak area (mAU) and concentration (µg/ml) using nine standard solutions of PADRE (Fig. 2).The linear equation of the standard curve and regression coefficientobtained was as follows:

Area = 29.12 (±0.17)* Concentration - 2.49 (±8.01)
(Correlation coefficient $[R]^2$) = 0.99

The calibration curve of PADRE showed a good linearity within the tested concentration range with a regression coefficient of 0.99 (13).

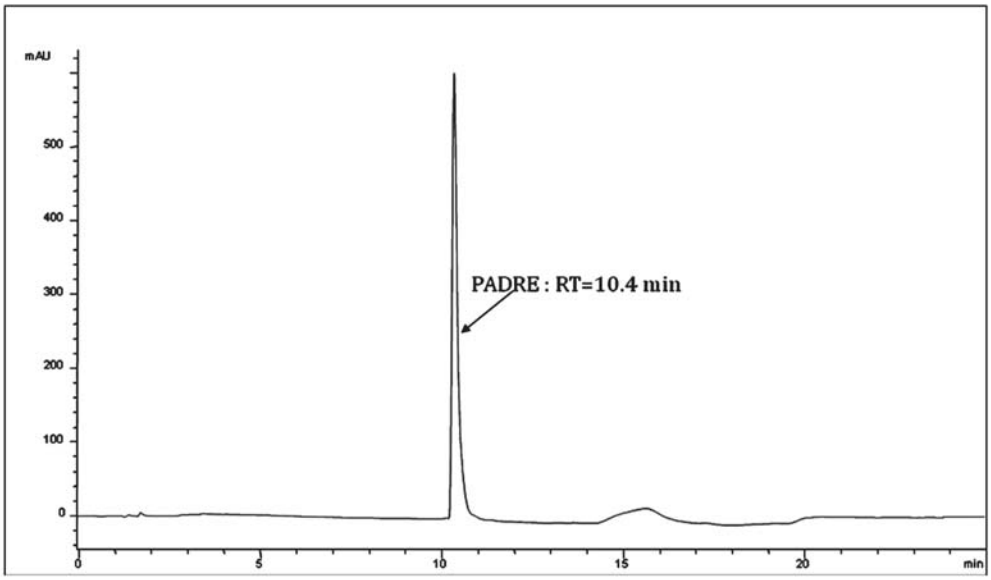

Fig. 1. Representative chromatogram for determination of immunogenic peptide PADRE

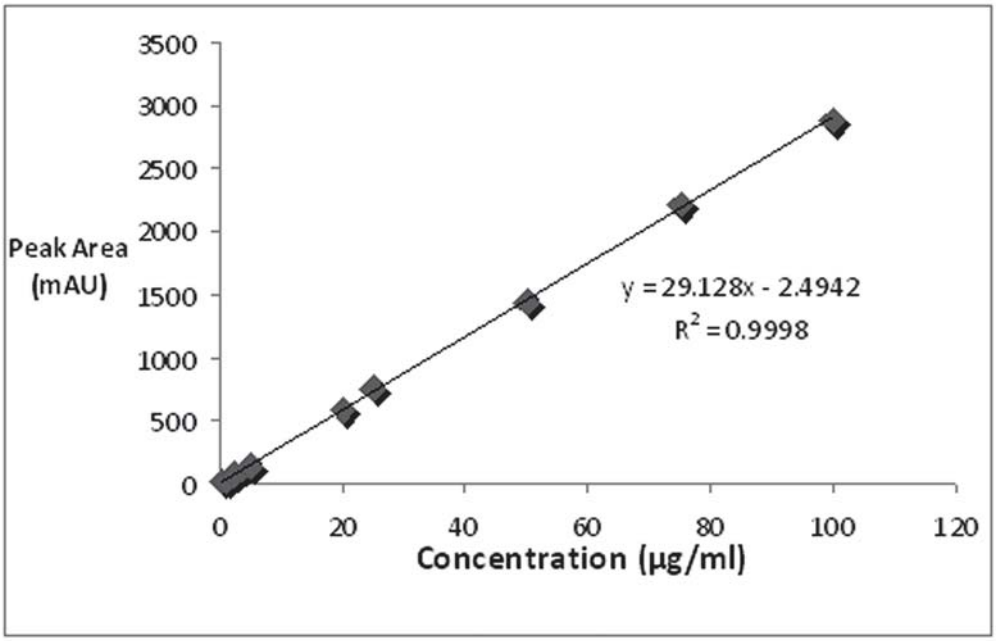

Fig. 2. Calibration curve obtained from standard solutions of PADRE by HPLC analysis

***Accuracyand Precision*:** The method was tested for accuracy, which is the closeness of the observed results to the actual results. The accuracy of the method is expressed as percent recovery calculated as follows:

$$\% \text{ Recovery} = \frac{\text{Observed Concentration}}{\text{Actual Concentration}} \times 100$$

From the results of the analysis (Table 1), the average recovery was observed to be 102, 97.80, 97.90, 104.64 and 99.50% at the standard concentrations of 1, 5, 10, 50 and 100 respectively. An overall average recovery of 100.36% was obtained. An average percentage recovery value between 95 to 105% is considered to be acceptable. Also no individual measurement of percentage recovery was less than 80% or greater than 120%. Thus the method was found to be accurate. Precision, which is a measure of repeatability of the data was also assessed for the method. The relative standard deviation (%RSD), also known as % coefficient of variation was determined by analyzing standard solutions of various concentrations on 5 consecutive days. The following formula was used to calculate the relative standard deviation:

$$\% \text{ RSD} = \frac{\text{Standard deviation of measurements}}{\text{Average of measurements}} \times 100$$

The variability improved at higher concentrations of the standard solutions.The %RSD was found to be reasonable for both intra-day and inter-day variability (Table 2). The proposed method for PADRE determination was found to be precise.

***Limit of detection (LOD) and limit of quantification (LOQ)*:** The limit of detection and

limit of quantification are measure of sensitivity of the method.The limit of quantification for the method at the tested concentrations of standards was found to be 0.5 µg/ml. At this concentration, both precision and accuracy were found to be less than 20%.The limit of detection, which is the lowest concentration of the analytethat can be detected by the proposed method was found to be 0.156 µg/ml.

RP-HPLC is a good technique to analyze peptides qualitatively and quantitatively due to the effective separation of peptides with nearly identical sequences. Enzymatic digestion products of peptides and proteins have previouslybeen effectively separated by RP-HPLC. For example,phosphorylation was utilized as an approach to improve the stability of an immunogenic peptide in serum.The stability of the unphosphorylated parent analogs; T 207-222, T 224-240, and T 390-408 and itsmonophosphorylated and diphosphorylated derivatives in serum was determined. The improvement in serum stability of phosphopeptides due to resistance of the serine and threonine phosphate ester bonds to serum proteases was linked to the improved immunogenicity of these peptides (14). The only disadvantage of using RP-HPLCfor peptide analysis is that, the peptide undergoes denaturation during the chromatographic run and therefore the method is not useful for preparative separations (15).

Application of the method

a) The analysispeptide PADRE after incubation in various biomatrices showed that the peptide was susceptible to degradation. Other than the peptide peak, three degradation peaks were observed in the chromatogram. PADRE showed extremely rapid degradation in plasma and therefore rate calculations could not be performed. The peptide degraded much more slowly if injected into the intact tumor mass and samples were taken from the medium but was much rapid in the presence of homogenized tumor. The peptide half-life was 30 minutes and 21 minutes in intact tumor and homogenized tumor, respectively (Fig.3). Thus the peptide

Table 1. Determination of accuracy for validation of the HPLC method for determination of PADRE (n=6)

Actual/ Prepared concentration (µg/ml)	Measured concentration (µg/ml)	Percentage Recovery (%)
1	1.02	102
5	4.89	97.80
10	9.79	97.90
50	52.32	104.64
100	99.50	99.50

Average percentage recovery (%) 100.368

Table 2. Determination of inter-day and intra-day variability to assessprecision of the HPLC method for determination of PADRE (n=6)

Actual concentration (µg/ml)	Relative standard % deviation (RSD)	
	Intra-day variability[1]	Inter-day varability[2]
1	2.6	10
5	3.8	8
10	1.6	3.7
50	1.2	2.2
100	2.1	1.9

[1]Intraday variability is determined by analyzing each concentration of standard solution 6 times within a day (n=6)

[2]Inter-day variability is determined by analyzing each concentration of standard solution on 5 consecutive days (n=5)

PADRE was relatively unstable in biomatrices. Several strategies have been proposed to improve plasma half-life of peptides (16) and these may be beneficial in improving the in vivo immnunogenic potential of PADRE.

b) PADRE was successfully entrapped in microparticles of PLGA. By HPLC analysis using the proposed method, the amount of PADRE at the surface of the particles was found to be 21.6± 2.2%. Similarly the amount of drug encapsulated within the PLGA matrix was found to be 27.2± 6.3%. Thus total peptide associated with the microparticles was found to be 54.3± 3.7%. Also, the release of PADRE was studied from the microparticles at two different levels of drug loading (1% and 5%w/w of peptide to polymer). The drug release (Fig. 4) was found to be biphasic, with an initial rapid phase and a

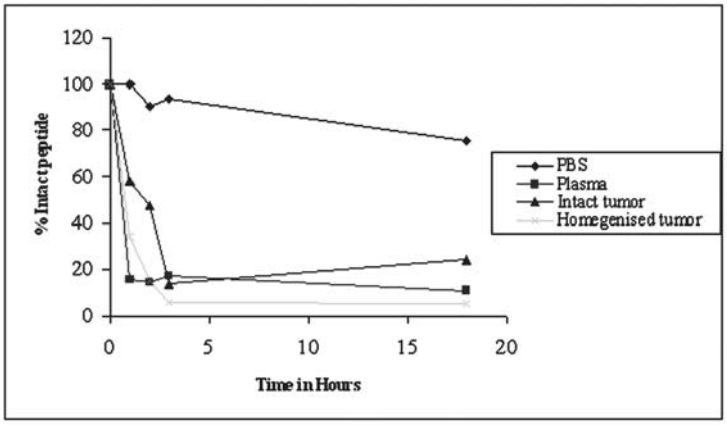

Fig. 3. Stability of the peptide PADRE in various biomatrices determined using the proposed HPLC method.

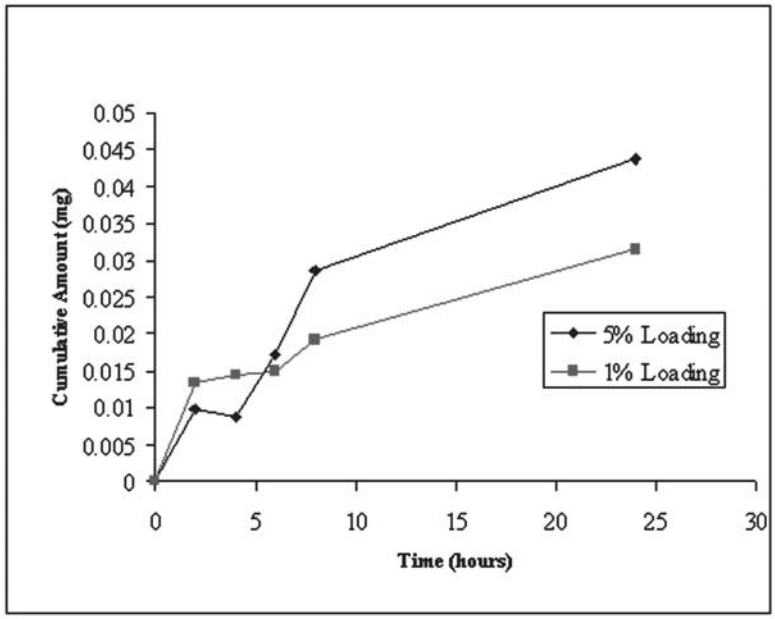

Fig. 4. Drug release of PADRE from PLGA microparticles

slow phase corresponding to the release of the encapsulated peptide.This pattern should mimic the profile of antigen concentrations that are seen in the course of a natural infection; i.e., a high dose of antigen within a few days of the injection followed by a period of decreasing amounts of antigen. The initial high load of antigen is expected to influence the extent of memory T-cell formation, whereas the subsequent steady decrease in antigen load will aid the development of antibody affinity maturation (17,18). PADRE release rate from the particles with 5% loading was higher when compared to the particles with 1% loading.

Conclusion

A reversed phase HPLC method was developed for the analysis of PADRE. The method was validated and found to be linear, precise, accurate and sensitive for detection of relatively low concentrations of analyte upto 0.5 µg/ml.The method was rapid with elution occurring at 10.4 minutes and runtime for each sample being 17 minutes. The proposed method was used to study stability of the peptide within biomatrices like plasma, tumor and tumor homogenates*in vitro*. Also, the method was used to study the encapsulation of PADRE within PLGA microparticles as drug delivery system and also used to characterize the subsequent release of PADRE from the microparticles. The method may further be applied to quantify PADRE at tumor site and in plasma upon systemic injection and also to study improvement in PADRE stability using enzyme inhibitors and various drug delivery systems.

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