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Method development and estimation of phenylenediamine in gastric contents, blood and urine

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Abstract:

A method for detecting and estimating Para-phenylenediamine (PPD) in the biological samples has been developed and validated using High-Performance Liquid Chromatography (HPLC) with UV/VIS detector. The method quantitatively measured the PPD in the biological samples. The mobile phase was composed of 10: 90 (Acetonitrile: Ammonium acetate 25 Mm v/v) of pH 4.5. The isocratic separation was carried out at C18 silica gel column at 1ml/minute of flow rate. The spectrophotometric detection was carried out at 240 nm. The process was validated, and the limit of detection for PPD was found to be 10µg. The accuracy and precision of the process were within limits. The linearity of the process found to be upto 1000 ppm. The extraction of PPD was carried out with chloroform and Sodium Hydroxide. The stock solution of PPD was freshly prepared due to oxidation potential. Aniline was used as an internal standard. The sample negative and sample positive were run to ensure the validity of the process. The estimation of the PPD was performed by drawing the calibration curve of different calibrators of PPD. The determination of PPD was carried out in gastric contents, Blood, and Urine. The extracted samples were prepared and run at HPLC for the detection. The pretreatment of the samples was not required, and the method proved to be accurate, precise, and specific.

Key words: Phenylenediamine; Pharmaceutical Science; Method development

Introduction

Para-Phenylenediamine (PPD), an organic compound and derivative of paranitroaniline compound has been traditionally used in dying and rubber industry, because of its oxidative chemical reaction PPD penetrates into hairs and produces colour to the hair. Accumulation of a large amount of the compound in the body may cause the following response such as skin, respiratory and ophthalmic reactions, moreover this synthetic compound will produce much life-threatening situation in many developing countries by producing intentional and unintentional intoxication (1).

It has been a long tradition to use such chemicals which are used to dye hairs and skin. The first synthetic hair dye was manufactured in

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1856. Hair dyes consist of the colorless developer, coupler and hydrogen peroxide an oxidising agent. PPD has been used commercially for more than a decade. These belong to permanent oxidative hair dyes that belong to a vast group of hair colourants (2). For the most part, p-phenylene-diamine (PPD), paminophenol, toluone-2,5-diamine or different arylamines are utilised as developers. Separated from being utilised within hair formulations PPD and its derivative serve as beginning materials for amalgamating azo dyes, antioxidants, tattoo colours and pharmaceuticals. Furthermore, they have sought colour photography and as quickening agents to synthesise polymer fibres (3).The primary purpose of this research is to build up the method and estimation of p-phenylenediamine in a biological sample by using HPLC

Materials and methods

This research project was performed at the Institute of Biochemistry and Biotechnology and WTO Laboratory, University of Veterinary and Animal Sciences, Lahore.

Experimental design: PPD in pure form is readily available in the local market. The PPD was orally administered to the rabbits, and biological samples (i.e. gastric fluid, heart, blood and urine) were collected by dissecting the test animal. The concentrations of samples were estimated by HPLC done by UV-Vis detector.The estimation is done for the concentration of paraphenylened-iamine in the biological samples. Thirty rabbits (19 male and 11 female) weighing from 0.95 kg – 1.70kg were selected as biological samples for the test. 250 mg/kg is the acute toxic dose of PPD in rabbits. Blood samples were drawn after 1 hour of ingestion of PPD to rabbits. Other biological samples,such as gastric fluid and urine,were collected from the rabbits immediately after death. Biological samples from the test animals (blood, urine and gastric content) were collected and was stored at biological refrigerator (-4 °C).

Chemicals and instrumentation: PPD Standard (Standpharm Pakistan (Pvt.) Ltd.), Aniline as IS (Standpharm Pakistan (Pvt.) Ltd.), Ammonium Acetate (Standpharm Pakistan (Pvt.) Ltd), Acetonitrile HPLC/Spectro Grade (M.W 41.1, Assay 99.9%, MP Biomedicals, LLC, France, HPLC grade Water, Distilled water. High Performance Liquid Chromatograph is used. Shimadzu HPLC system fitted with autosampler (SIL-20A), reciprocating pump (LC-20AT), and solvent delivery system (LC-20A) with system controller (CBM-20A), detection was done by a UV-Vis detector at wavelength of 240 nm and Merck C18 column (15 cm x 3.9 mm x 5u) was used.

Preparation of standard: PPD 1000 mg was dissolved in 500 mL of distilled water to make 1000 ppm solution and make up the final volume 1000 mL in the volumetric flask. The stock solution was freshly prepared daily because of the oxidative degradation of the PPD.A dilution of 20-120ppm solutions were prepared to make the calibration curve.Aniline was used as Internal Standard. It is not present in biological samples. Further, it produced reproducible results.Aniline (IS) 100 mg was dissolved in 50 mL of distilled water to make 1000 ppm solution and made up the final volume 100 mL in the volumetric flask.To prepare 100 mL of 500 ppm solution, 50 mL of the stock IS solution was diluted with 30 mL of mobile phase in a volumetric flask and, in the end, the final volume made up to 100 mL with the mobile phase. The retention time of target analyte (PPD) and internal standard (Aniline) was determined by running 100 ppm solutions of each, separately, on HPLC.

Preparation of positive control: To prepare 100 mL of 0.2% w/v positive quality control, 200 mg of PPD standard was accurately weighed and dissolved in 50 mL 10 ml Acetonitrile solution and make the volume to 100 ml by Ammonium acetate buffer. The solution was refrigerated before use. This was a reference solution or test solution used for assessment of the performance of the procedure.

Preparation of negative control: Acetonitrile 10 % solution and ammonium acetate 90% (25Mm)

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buffer was used as a negative control sample. It was PPD free sample. This was a reference solution or test solution used for assessment of the performance of the procedure.

Preparation of samples:

Gastric contents sample preparation: The organic extraction procedure was employed for the preparation of the sample for the detection of PPD. 2ml of sample is added with 2ml chloroform and 100 microliter internal standard mixed for 15 minutes, then separate the organic layer. Repeat the same procedure for further purification and complete extraction without an internal standard for 3 times. The organic layer was dried by nitrogen purging. Mix the dried extract with the mobile phase. The solution was further processed for the estimation of PPD.

Blood sample preparation: The 2mL whole blood sample is added with 100 microliter of internal standard and was subjected to centrifugation. The plasma of the sample is added with chloroform, and the extraction proceeded. The extracted sample is dried under nitrogen and reconstituted with the mobile phase. This solution was further processed for the estimation of PPD.

Urine sample preparation: 2mL urine sample 2 mL, is added with 100 microliter of internal standard, 0.1 N NaOH and 2ml of chloroform. The solution was mixed for 15 minutes. The organic layer separated was added with chloroform; the procedure is repeated thrice. The extracted sample was then dried by nitrogen purging. The sample was reconstituted with the mobile phase. This solution was further processed for the estimation of PPD.

Procedure: The method for the detection of PPD in biological samples was optimised. Extraction was performed after the addition of Internal Standard. 10ml volume of all calibrator, Positive QC, Negative QC and all samples were pipette out in 15 mL centrifuge tubes. This produced the final strength of 100 ppm of IS. The extraction procedure was done by the addition of chloroform and 0.1N NaOH and mixed for 15 minutes. The chloroform layer was separated and purified by repeating the extraction for 3 times. The extracts were dried under nitrogen gas. The dried extracts were dissolved in 10 mL of mobile phase and ran on HPLC.

Preparation of mobile phase: Mobile phase was composed of Acetonitrile: Ammonium acetate solution (10:90). The Ammonium acetate solution was of 25mM strength having pH of 6.5. Scientific Working Group for forensic Toxicology (SWGTOX) guidelines for validation of quantitative analysis was followed. SWGTOX is the group of forensic toxicology experts from America and another world who set forensic toxicology laboratories standards in the world (4). The following validation parameters were evaluated:

Linearity and calibration model: The linearity/ calibration samples were prepared using simulated gastric contents. The concentrations prepared were 0.04mg/g, 0.24mg/g, 0.48mg/g, 2.38mg/g, 4.75mg/g, and 23.7mg/g of zinc phosphide equivalent to 10µg/g, 50µg/g, 100µg/g, 500µg/g, 1000µg/g and 5000µg/g of PPD respectively. Each concentration level was evaluated in five different runs on, and concentration versus mean area curve for each concentration was drawn.

Accuracy and precision: Three levels were selected to establish accuracy and precision. These levels were prepared by fortifying the blank gastric contents at concentrations 50µg/g, 100µg/g and 1000µg/g.Different concentrations of PPD ranging from 0.5ug/g to 50ug/g were prepared by spiking the appropriate amount of PPD in simulated gastric contents and run on HPLC to find LOD.Various concentrations of PPD from 1µg/g to 50µg/g were prepared by spiking PPD into the simulated gastric contents and run on HPLC to find LOQ. Guidelines were taken from quality control analytical methods (5).

Chromatographic conditions: Shimadzu HPLC system fitted with autosampler (SIL-20A), reciprocating pump (LC-20AT) and Merck C18 column (15 cm x 3.9 mm x 5u) was used for

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separation (6)(7). A Shimadzu HPLC solvent delivery system controller (LC-20A) with system controller (CBM-20A) was used. The 20µL sample was used in the injection system. Detection was done by UV-Vis detector at a wavelength of 240 nm.The instrument's response to increasing concentrations of analyte was found to be linear over the range of 10µg/g to 1000µg/g. However, the linearity was broken above the 1000µg/g of PPD. The peak area for each concentration in five different runs is given in the table below.

Results

The linearity study results indicated that the method provides a linear response within the range of the most probable amount of PPD in the stomach contents (Figure 1).When unintended analyte appears in the blank sample after analysingthe positive sample, it is called carryover. Carryover is evaluated during method validation for quantitative as well as qualitative analysis. It was found that no carryover was observed up to the concentration of 1000µg/g of PPD. However, little carryover peak was observed after the 5000µg/g of p7 PPD level but its integrated area was less than 10% of the smallest area of 10µg/g level.

Accuracy studies are usually conducted in concurrent with precision studies. At least three levels are selected at low, medium and high concentrations, and fortified blank samples of these levels are analysed for five days. Following formula was used to calculate accuracy at each concentration level.

$$\text{Accuracy at Concentration}_x = \left[\frac{\text{Grand Mean of Calculated Concentration}_x - \text{Known Concentration}_x}{\text{Known Concentration}_x}\right] \times 100$$

The results of measured amounts of each concentration level are presented in the table below (Table 2). Accuracy and precision for each concentration level were calculated from the mean measured amount of the five days' data.

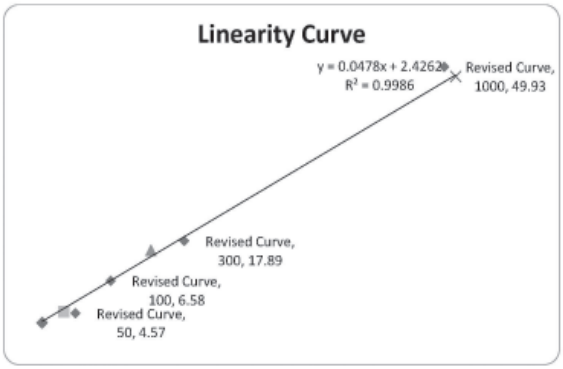

Fig 1: Validated Linearity Curve

Table 1: Data for Accuracy and Precision Studies

Days	50 µg/g	100 µg/g	1000 µg/g
1	44.5	129	997.3
2	53	83.9	997.5
3	41.1	125.7	684.8
4	60	101.1	989.5
5	37	118.4	944.1
Mean	47.2	111.6	922.64
Standard deviation	8.5	18.87	135

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Accuracy at concentration 50µg/g:

$$Accuracy_{50} \quad \text{———} \times 100$$

$$= -5.6\% \text{ or } 94.4\ \%$$

Accuracy at concentration 100 µg/g:

$$Accuracy_{100} = \text{———} \times 100$$

$$= +11.6\ \% \text{ or } 116\%$$

Accuracy at concentration 1000 µg/g:

$$Accuracy_{1000} = \text{———} \times 100$$

$$= -7.73\ \% \text{ or } 92.3\ \%$$

$$Between - Run\ CV(\%) = \frac{\sigma\ of\ grand\ mean\ for\ each\ level}{grand\ mean\ for\ each\ level} x100$$

σ is the standard deviation of the response of one level.

$$Precision_{50} = \text{——} \times 100 \quad = 18\ \%$$

$$Precision_{100} = \text{——} \times 100 \quad = 16.9\ \%$$

$$Precision_{1000} = \text{——} \times 100 \quad = 14.6\ \%$$

Limit of detection and limit of quantification: Limit of detection (LOD) is the minimum amount of the analyte that can be detected by analytical procedure (8). In the study the LOD was found to be 10µg/g. The peak produced at the retention time of PPD i.e.; 2.317 minutes was reproducible at the concentration of 10µg/g. below this concentration, the response was not reproducible.Limit of Quantification is the minimum concentration that can be quantitated, and method can produce multiple symmetrical results. 1000µg/g was the minimum concentration at which the multiple runs produced symmetrical peaks, and the results were within ±20% of accuracy and %CV of less than 20 %.So the LOQ found to be was 1000ug/g.

Retention time measurement: 100 ppm of PPD solution was run, and it gave a retention time of 2.320 minutes. Further, 100 ppm solution of Aniline (internal standard) produced a peak at 15.660 minutes showing its retention time.Negative QC gave a retention time of 5.287 min and did not show PPD peak. 0.2% w/v PPD solution was run, and it gave retention time of 8.665 min, and Aniline (internal standard) produced a peak at 5.337 min showing its retention time. Sample AHZ B1 was Blood. Its chromatographic profile shows that AUC of the PPD is 46797465 and that of (IS) is 16445487.Sample AHZ B2 was Blood. Its

Table 2: Summary of validation results

Parameter	Desired limit	Results
Calibration Model	50-1000 µg/g (linear model desired)	50-1000µg/g Linear model
Accuracy	Must not exceed ±20%	92.3 to111.6 %
Precision	%CV must not exceed 20%	14.6 to18 %
Limit of Detection (LOD)	Must be 3 µg/g or lower	10 µg/g
Limit of Quantitation (LOQ)	Must be 10 µg/g or lower	1000 µg/g
Carryover	Carryover after highest calibrator does not exceed 10% of signal of lowest calibrator	No carryover

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chromatographic profile shows that AUC of the PPD is 46906162 and that of (IS) is 15113134.Sample AHZ B3 was Blood. Its chromatographic profile shows that AUC of the PPD is 30134559 and that of (IS) is 14127024.Sample AHZ B4 was Blood. Its chromatographic profile shows that AUC of the PPD is 30014839 and that of (IS) is14221388.Sample AHZ B5 was Blood. Its chromatographic profile shows that AUC of the PPD is 30148627 and that of (IS) is 14570160.Sample AHZ B6 was Blood. Its chromatographic profile shows that AUC of the PPD is 48825438 and that of (IS) is14189546.Sample AHZ B7 was Blood. Its chromatographic profile shows that AUC of the PPD is 44797777 and that of (IS) is 16156445.Sample AHZ B8 was Blood. Its chromatographic profile shows that AUC of the PPD is 46747497 and that of (IS) is16245444.Sample AHZ B9 was Blood. Its chromatographic profile shows that AUC of the PPD is 46579877 and that of (IS) is16478944.Sample AHZ B10 was Blood. Its chromatographic profile shows that AUC of the PPD is 46787877 and that of (IS) is 16777224.Sample AHZ G11 was gastric content. Its chromatographic profile shows that AUC of the PPD is 48785968and that of (IS) is16030762.Sample AHZ G12 was gastric content. Its chromatographic profile shows that AUC of the PPD is 41796895 and that of (IS) is 16638495.

Sample AHZ G13 was gastric content. Its chromatographic profile shows that AUC of the PPD is 43977751 and that of (IS) is 16916781.Sample AHZ G14 was gastric content. Its chromatographic profile shows that AUC of the PPD is 49663343 and that of (IS) is 15690167.Sample AHZ G15 was gastric content. Its chromatographic profile shows that AUC of the PPD is 45457874 and that of (IS) is 16979794.Sample AHZ G16 was gastric content. Its chromatographic profile shows that AUC of the PPD is 46549498 and that of (IS) is 16444654.Sample AHZ G17 was gastric content. Its chromatographic profile shows that AUC of the PPD is 723797 and that of (IS) is 1438402.Sample AHZ G18 was gastric content. Its chromatographic profile shows that AUC of the PPD is 189870 and that of (IS) is 1448288.Sample AHZ G19 was gastric content. Its chromatographic profile shows that AUC of the PPD is 1836744 and that of (IS) is 1487337.Sample AHZ G20 was gastric content. Its chromatographic profile shows that AUC of the PPD is 821681 and that of (IS) is 1300566.Sample AHZ U21 was urine. Its chromatographic profile shows that AUC of the PPD is 985347 and that of (IS) is 1413061.Sample AHZ U22 was urine. Its chromatographic profile shows that AUC of the PPD is 94776 and that of (IS) is 1485271.Sample AHZ U23 was urine. Its chromatographic profile shows that AUC of the PPD is 2546 and that of (IS) is 1534889.Sample AHZ U24 was urine. Its chromatographic profile shows that AUC of the PPD is 2546 and that of (IS) is 1534889.Sample AHZ U25 was urine. Its chromatographic profile shows that AUC of the PPD is 1040184 and that of (IS) is 1200902.Sample AHZ U26 was urine. Its chromatographic profile shows that AUC of the PPD is 749238 and that of (IS) is 1787173.Sample AHZ U26 was urine. Its chromatographic profile shows that AUC of the PPD is 749238 and that of (IS) is 1787173.Sample AHZ U28 was urine. Its chromatographic profile shows that AUC of the PPD is 749238 and that of (IS) is 1787173.Sample AHZ U29 was urine. Its chromatographic profile shows that AUC of the PPD is 1054736 and that of (IS) is 1693898.Sample AHZ U30 was urine. Its chromatographic profile shows that AUC of the PPD is 1149796 and that of (IS) is 1692019.

Discussion

Mainly, para-phenylenediamine (PPD), paminophenol, toluone-2,5-diamine or other arylamines are used as developers. Apart from being used in hair formulations PPD and its derivatives serve as starting materials to synthesise azo dyes, antioxidants, tattoo colours and pharmaceuticals. In addition, they are applied for colour photography and as accelerators for the synthesis of polymer fibres (3).

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Table 3: Standard Deviation From The Mean In Blood Samples

Sample	AUC	Regression constant (a)	Regression constant (b)	Concentration of PPD (ppm)	SD
B1	46809018	289210	2.00E+06	154.9359	
B2	46909192	289210	2.00E+06	155.2823	
B3	30134559	289210	2.00E+06	97.28073	
B4	30014839	289210	2.00E+06	96.86677	
B5	30148627	289210	2.00E+06	97.32937	**29.94086**
B5	30148627	289210	2.00E+06	97.32937	
B6	48825438	289210	2.00E+06	161.9081	
B7	44797777	289210	2.00E+06	147.9817	
B8	46747497	289210	2.00E+06	154.7232	
B9	46579877	289210	2.00E+06	154.1436	
B10	46909492	289210	2.00E+06	155.2833	

Table 4: Standard Deviation From The Mean In Urine Samples

Sample	AUC	Regression constant (a)	Regression constant (b)	Concentration of PPD (ppm)	SD
U1	32473823	289210	2.00E+06	105.3692	
U2	20040976	289210	2.00E+06	62.38019	
U3	32786089	289210	2.00E+06	106.4489	
U4	28726878	289210	2.00E+06	92.4134	
U5	29955139	289210	2.00E+06	96.66035	**22.90191**
U6	20644454	289210	2.00E+06	64.46684	
U7	19899494	289210	2.00E+06	61.89099	
U8	20465414	289210	2.00E+06	63.84777	
U9	16859794	289210	2.00E+06	51.38064	
U10	14954544	289210	2.00E+06	44.79286	

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Table 5: Standard Deviation From The Mean In Gastric Samples

Sample	AUC	Regression constant (a)	Regression constant (b)	Concentration of PPD (ppm)	SD
G1	48785968	289210	2.00E+06	161.7716	
G2	51764161	289210	2.00E+06	172.0693	
G3	41796895	289210	2.00E+06	137.6055	
G4	43977751	289210	2.00E+06	145.1463	
G5	49663343	289210	2.00E+06	164.8053	**10.09062**
G6	45457874	289210	2.00E+06	150.2641	
G7	46549498	289210	2.00E+06	154.0386	
G8	47987465	289210	2.00E+06	159.0106	
G9	46564445	289210	2.00E+06	154.0903	
G10	48987945	289210	2.00E+06	162.47	

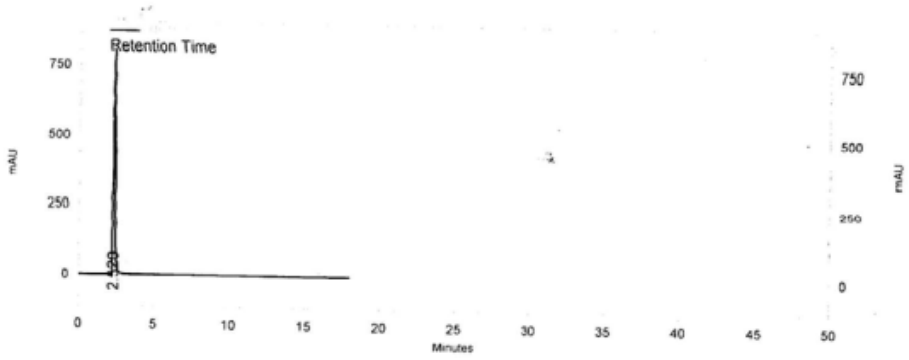

Fig 2: Chromatogram of Retention time of PPD

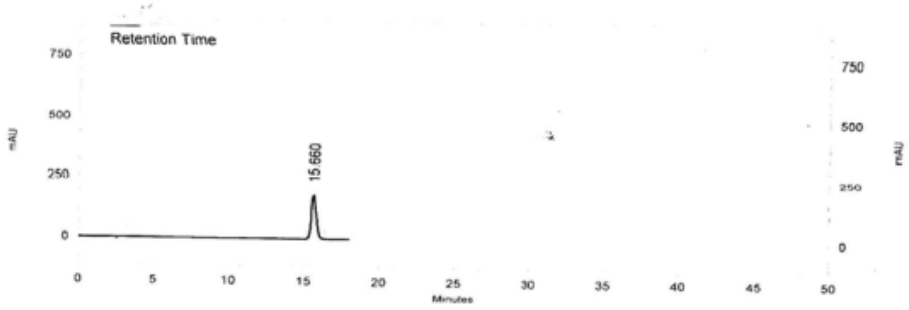

Fig 3: Chromatogram of Retention time of Aniline

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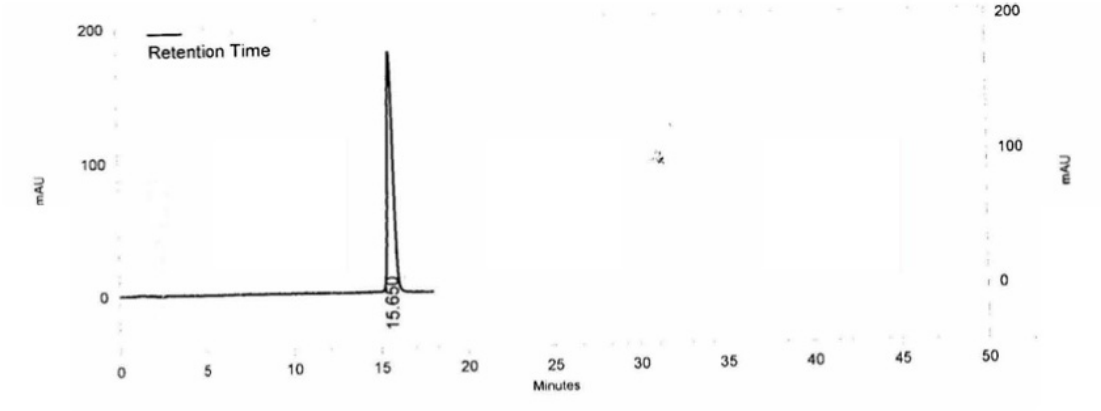

Fig 4: Chromatogram of Negative Quality Control

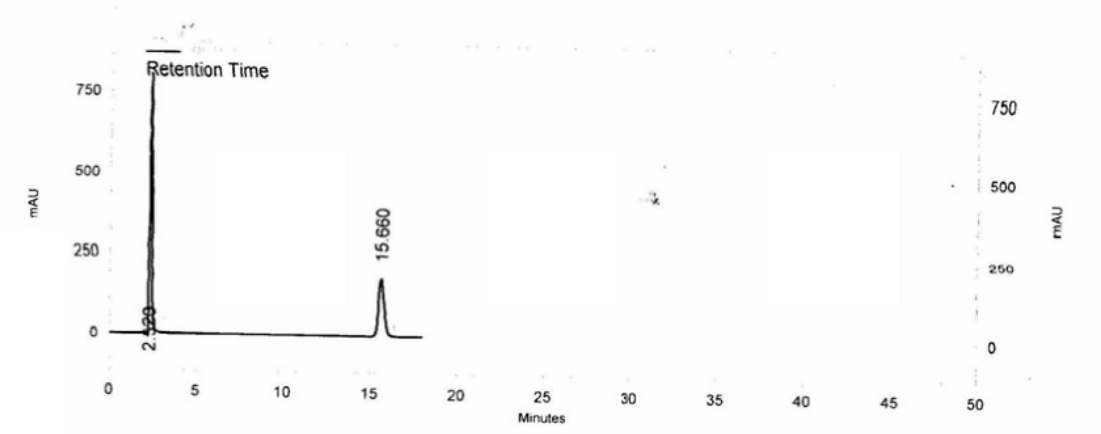

Fig 5: Chromatogram of Positive Quality Control

According to Axel Meyer at el 2009 (9) developed a method for the quantitative detection of PPD using HPLC. The mobile phase was composed of acetonitrile; ammonium acetate buffer(5:95, 25mM v/v). The limit of quantification of PPD was determined at 0.5 µM for PPD. In the present study,a method for the detection and estimation of Para phenylenediamine in the biological samples has been developed and validated using HPLC UV/VIS. The method could quantitatively measure the PPD in the biological samples. The mobile phase was 10: 90 (ACN: ammonium acetate 25 Mm v/v) of pH 4.5.The isocratic separation was carried out at C18 silica gel column at 1ml/minute of flow rate. The spectrophotometric detection was carried out at

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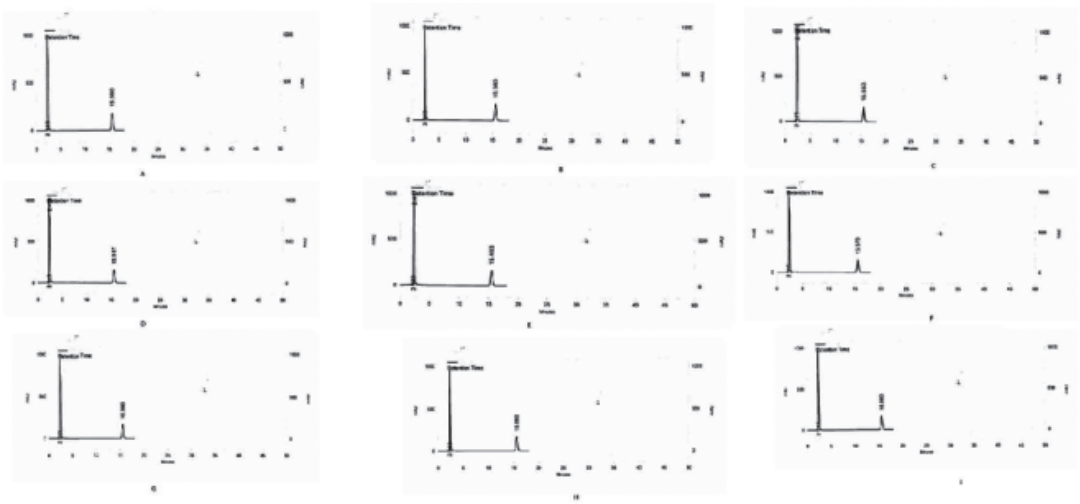

Fig 6: Chromatographic profile of samples (A)AHZ B1 (B) AHZ B2 (C) AHZ B3 (D) AHZ B4 (E) AHZ B5 (F) AHZ B6 (G) AHZ B7 (H) AHZ B8 (I) AHZ B9

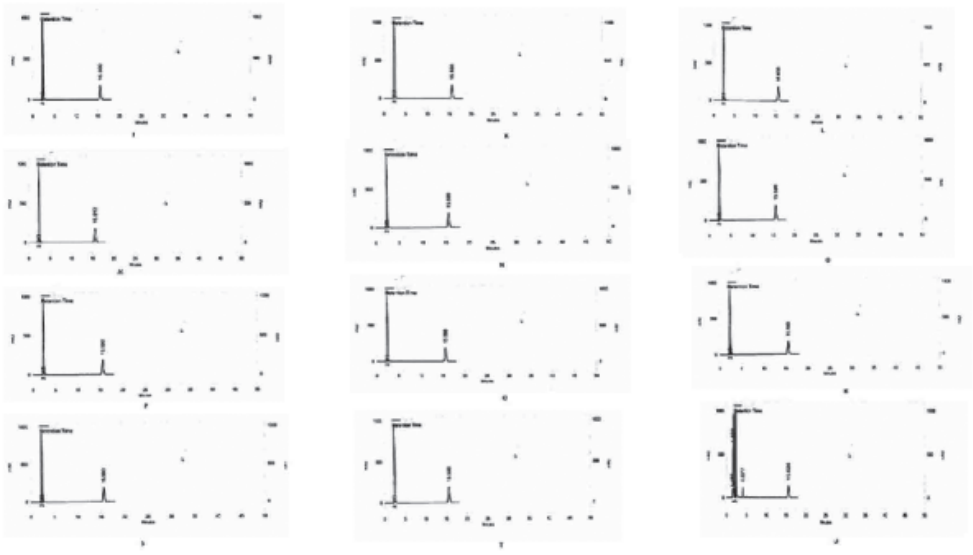

Fig 7: Chromatographic profile of samples (J)AHZ B10 (K) AHZ B11 (L) AHZ B12 (M) AHZ B13 (N) AHZ B14 (O) AHZ B15 (P) AHZ B16 (Q) AHZ B17 (R) AHZ B18 (S) AHZ B19 (T) AHZ B20 (U) AHZ B21

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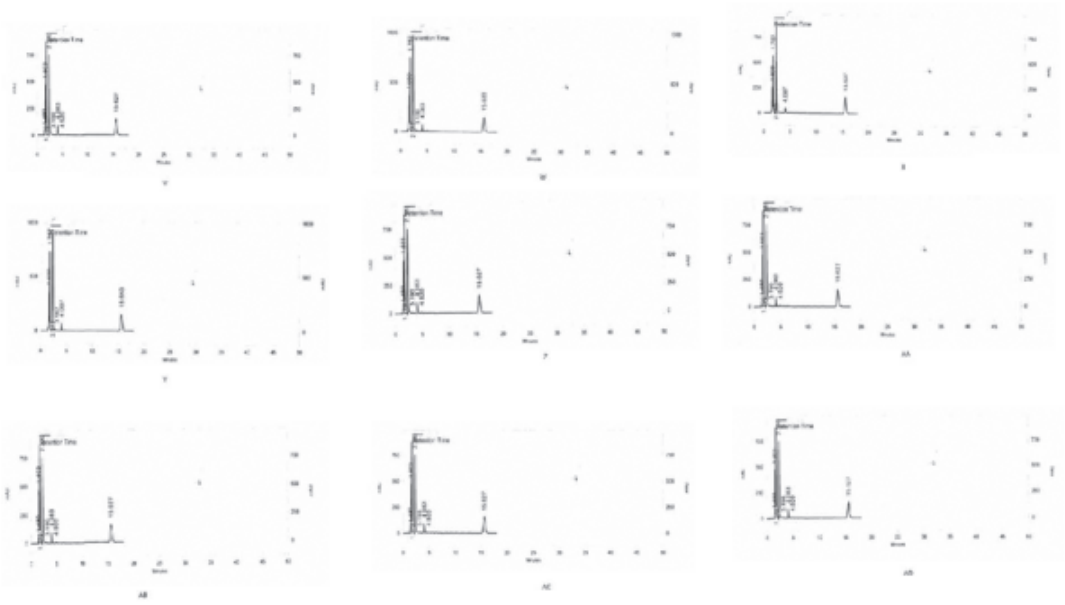

Fig 8: Chromatographic profile of samples (V)AHZ B22 (W) AHZ B23 (X) AHZ B24 (Y) AHZ B25 (Z) AHZ B26 (AA) AHZ B27 (AB) AHZ B28 (AC) AHZ B29 (AD) AHZB30

240 nm. The process was validated, and the limit of detection for PPD was found to be 10µg. The accuracy and precision of the process was within the limits. The linearity of the process found to be upto 1000 ppm. The interday and intraday precision and accuracy were withinlimits.The extraction of PPD was carried out with chloroform and NaOH. The Stock solution of PPD was freshly prepared due to oxidation potential. Aniline was used as an internal standard. The sample negative and sample positive were run to ensure the validity of the process. The estimation of the PPD was performed by drawing the calibration curve of different calibrators of PPD. The determination of PPD was carried out in Gastric contents, Blood, and Urine. The extracted samples were prepared and run at HPLC for the detection. The pretreatment of the samples was not required, and the method proved to be accurate, precise, and specific.

References

1. Hooff, G. P., van Huizen, N. A., Meesters, R. J., Zijlstra, E. E., Abdelraheem, M., Abdelraheem, W., ... & Luider, T. M. (2011). Analytical investigations of toxic p-phenylenediamine (PPD) levels in clinical urine samples with special focus on MALDI-MS/MS. PloS one, 6(8), e22191.

2. Corbett, J. F. (1973). Hair colouring. Review of Progress in Coloration and Related Topics, 4(1), 3-7.

3. J. Hansen, B. Møllgaard, C. Avnstorp, and T. Menné, “Paraben Contact Allergy: Patch Testing and In Vitro Absorption/ Metabolism,”*Dermatitis*, vol. 4, no. 2, 1993.

4. Scientific Working Group for Forensic Toxicology. (2013). Scientific Working

Vol. 15 (1) 22-33 January 2021, ISSN 0973-8916 (Print), 2230-7303 (Online)
DOI: 10.5530/ctbp.2021.1.3

Group for Forensic Toxicology (SWGTOX) standard practices for method validation in forensic toxicology. Journal of Analytical Toxicology, 37(7), 452-474.

5. Kupiec, T. (2004). Quality-control analytical methods: High-performance liquid chromatography. International journal of pharmaceutical compounding, 8, 223-227.

6. Almeida, P. J., Borrego, L., Pulido Melián, E., & González Díaz, O. (2012). Quantification of p phenylenediamine and 2 hydroxy 1, 4 naphthoquinone in henna tattoos. *Contact dermatitis*, *66*(1), 33-37.

7. Sadiq, M.A., Zohra, E., Jamil, M.A., Wasim, M., Riaz, H., Raza, S.A., Aslam, M.S., Hussain, S., Javed, O. and Ahmad, M.A., 2018. Estimation of Caffeine Concentration in Decaffeinated Coffee and Tea Available in Pakistan. Journal of Pure and Applied Microbiology, 12(1), pp.229-247.

8. Armbruster, D. A., & Pry, T. (2008). limit of blank, limit of detection and limit of quantitation. *The clinical biochemist reviews*, *29*(Suppl 1), S49.

9. Meyer, A., Blömeke, B., & Fischer, K. (2009). determination of p-phenylene diamine and its metabolites MAPPD and DAPPD in biological samples using HPLC-DAD and amperometric detection. *Journal of Chromatography B*, *877*(16-17), 1627-1633.