Development and Validation of an LC–MS/MS Method for Determination of *p*-Phenylenediamine and its Metabolites in Blood Samples

Khaled M. Mohamed[1], Duncan Cromarty[2] and Vanessa Steenkamp[2]

[1]Assiut Chemical Laboratory, Medico-Legal Department, Ministry of Justice, Assiut, Egypt

[2]Department of Pharmacology, Faculty of Health Sciences, University of Pretoria, Pretoria, South Africa

Corresponding author contact: khaled.masoud@yahoo.com, Tel: 00201003100517, Fax: 0020882324038

Abstract

In some developing countries, *p*-phenylenediamine (PPD) is used in combination with Henna as hair dye or skin decoration. A sensitive LC–MS/MS method was developed and validated for the simultaneous determination of *p*-phenylenediamine (PPD) and its metabolites *N*-acetyl-*p*-phenylenediamine (MAPPD) and *N*,*N*-diacetyl-*p*-phenylenediamine (DAPPD) in human blood. Acetanilide was used as an internal standard (IS). The LC-MS/MS was operated under multiple reaction-monitoring mode using the electrospray positive ionization technique. The transition ions m/z 109 → 92, m/z 151 → 92, m/z 193 → 92, and m/z 136 → 77 were selected for the quantification of PPD, MAPPD, DAPPD, and IS, respectively. The linear range was 10–2000 ng/mL for all the compounds. The absolute recoveries were 51.94, 56.20 and 54.88% for PPD, MAPPD and DAPPD, respectively. Intra- and inter-assay imprecision were lower than 14% (RSD), and the bias of the assay was lower than 15% for all the compounds. The stability studies demonstrated that critical degradation for PPD in blood samples and autosampler occurred after 6 h, while MAPPD and DAPPD were stable in blood samples and the autosampler up to 48 h and 24 h, respectively. This newly developed method allows for the detection of PPD and its metabolites in blood samples in the clinical and forensic setting.

Keywords: *p*-Phenylenediamine; Metabolites; Blood; LC-MS/MS; Validation; Stability

1. Introduction

p-phenylenediamine (PPD) is an azo dye intermediate used for dying furs, photochemical measurements, as a photographic developing agent, as an intermediate in manufacture of antioxidants and as accelerators for rubbers. In some African and Asian countries, this chemical is used alone or in combination with Henna for dyeing of hair and skin [1-3]. A vast numbers of suicidal, homicidal and accidental poisoning cases involving PPD have been recorded [4-7].

In 20 fatal p-phenylenediamine poisoning cases, convulsion, facial edema and cyanosis were characteristic whereas edemas of the epiglottis and vocal folds were observed in all cases [8] change number. Other poisoning symptoms after oral intake include vomiting, epigastralgia, edema of the neck and pharynx, dyspnea, acute renal failure, rhabdomyolysis, hemolysis, methemoglobinemia and hepatic failure [8-10]. PPD is rapidly absorbed into blood through mucous membranes of the digestive tract after its oral intake, and metabolized into quinonediimine, which acts as a cytotoxin. It is acetylated into *N*-acetyl-p-phenylenediamine (MAPPD) and *N*,*N*-diacetyl-*p*phenylenediamine (DAPPD) as the major metabolites for detoxification to be excreted into urine [11-13].

PPD has been detected in blood, urine and gastric content by gas chromatography–mass spectrometry (GC–MS) after liquid–liquid extraction [14,15]. Methods involving liquid chromatography (LC) with electrochemical detector [16], ultraviolet [16,17], diode array [18], or MALDI-MS/MS [17] for quantification of PPD and its metabolites have been reported. LC-MS/MS is currently the classical analytical tool in forensic and clinical laboratories for the analysis of most of the common drugs and toxic substances in biological matrices.

A validated LC–MS/MS method for detection and quantification of PPD and its metabolites in human blood has not been described to date. Therefore, the aim of this study was to a sensitive LC–MS/MS method for the simultaneous determination of PPD, MAPPD and DAPPD in human blood. The method was successfully applied to analysis of human blood samples collected from postmortem cases provided by Assiut Forensic Chemistry Laboratory of Medico-Legal Department, Ministry of Justice, Egypt.

2. Materials and methods

2.1. Reagents and standards

p-Phenylenediamine dihydrochloride (99%), *N*-acetyl-p-phenylenediamine (99%), *tert.*-Butyl methyl ether (99.8) and acetanilide (99%) were purchased from Sigma-Aldrich. *N*,*N*-diacetyl-p-phenylenediamine (DAPPD) was synthesized in our laboratory [13]. Formic acid (98%), ammonium acetate (98%) and ammonium formate (99%) were purchased from Fluka Chemie GmbH. Acetonitrile (99.9%) and ammonium hydroxide (25%) were purchased from Merck. Methylene chloride (HPLC) and Ethly acetate (HPLC) were purchased from Baxter. Methanol (99.9%) was purchased from Romil pure Chemistry.

2.2. Instrumentation

An Agilent LC 1100 binary pump, autosampler, vacuum degasser, column oven (Agilent Technologies), and a Eclipse XDB C18 column (150 mm x 4.6 mm, 5µ) were carried out for chromatographic separations. The mass spectrometric analysis was performed by use of an AB/MDS Sciex 4000 QTrap LC–MS/MS (Applied Biosystems, Canada) instrument in triple quadrupole mode, equipped with an AB/MDS Sciex Turbo Ion Spray interface. The software was Analyst 1.4.1.

2.3. Liquid chromatography

The analytical column was maintained at 25 ^{0}C. A mobile phase gradient pumped at 1 mL/min was used to elute the analytes from the column. Mobile phase A consisted of acetonitrile. Mobile phase B consisted of 0.1% formic acid. The gradient was initiated at 100% B for 2 min, and subsequently a linear gradient led to 70% B in 1 min, kept for 2.75 min and brought back to 100% B in 0.25 min. The column was equilibrated for 2 min. Total run time was 8 min. Injection volume was 10 µL.

2.4. Mass spectrometry

Detection of analytes and IS was performed on a triple quadrupole mass spectrometer operating in the positive mode (ESI^{+}) with multiple reaction monitoring (MRM). The most abundant fragment for each compound was selected by performing enhanced product ion scans of the standards during an infusion analysis using a Harvard syringe pump at a constant flow rate of 10µL/min. For each compound, two or three mass

Table 2
Summary of calibration curves for PPD, MAPPD and DAPPD in human blood (n = 3) with back calculated concentrations.

	Concentration (ng/mL)						Regression Equation	
	10	50	100	500	1000	2000	Slope	r^2
PPD								
Mean	10.6	53.9	108.3	519.3	971.7	1960.0	0.0140	0.9853
SD	0.3	3.8	4.0	35.5	59.7	113.6	0.0069	0.0127
% RSD	2.9	7.1	3.7	6.8	6.1	5.8	0.4929	0.0129
%Bias	5.7	7.9	8.3	3.9	-2.8	-2.0		
MAPPD								
Mean	10.3	49.0	109.3	522.7	1008.3	2086.7	0.0196	0.9807
SD	0.6	1.8	5.0	32.1	28.4	181.5	0.0060	0.0083
% RSD	5.5	3.7	4.6	6.1	2.8	8.7	0.3049	0.0084
% Bias	2.7	-1.9	9.3	4.5	0.8	4.3		
DAPPD								
Mean	11.1	48.7	110.0	520.7	1022.0	2060.0	0.0099	0.9897
SD	0.4	4.1	6.1	26.1	87.0	120.0	0.0021	0.0154
% RSD	3.9	8.4	5.5	5.0	8.5	5.8	0.2073	0.0155
% Bias	11.0	-2.6	10.0	4.1	2.2	3.0		

SD: Standard deviation
RSD: Relative standard deviation
r^2: Correlation coefficient

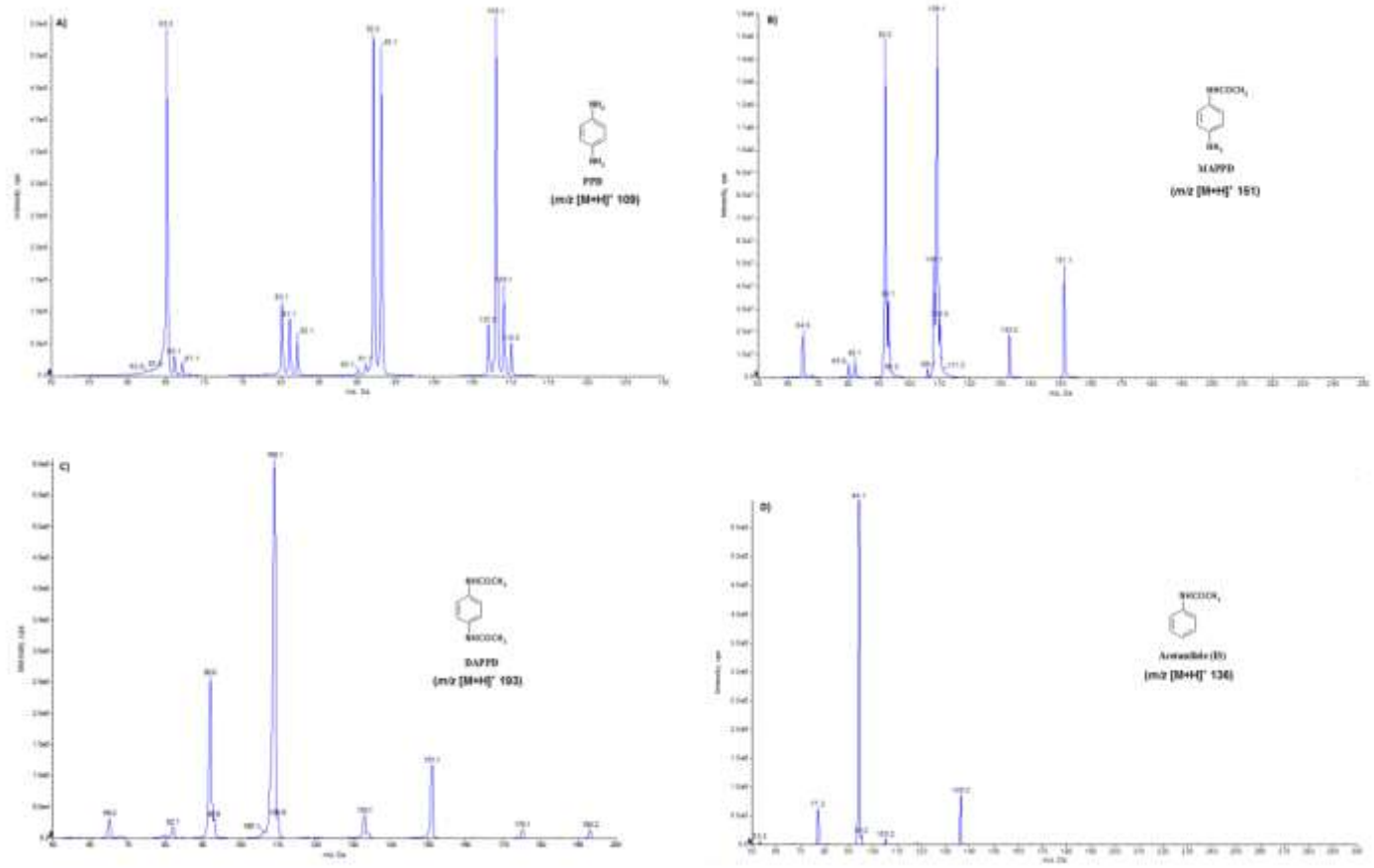

Fig 1. MS–MS spectra in the positive mode (ESI^+) of the precursor ions at m/z 109 for PPD (A), m/z 151 for MAPPD (B), m/z 193 for DAPPD (C), and m/z 136 for IS (D).

fragments were monitored with one fragment used for quantification and the other fragments used for the additional confirmation of identity (Fig. 1). The MRM transitions are reflected in Table 1. The compound dependent parameters like the collision energy (CE) and declustering potential (DP) were adjusted to provide the highest sensitivity (Table 1). Compound independent parameters that remained constant were as follows: curtain gas (CUR): 23 psi; Ion spray voltage (ISV): 5500 V; Ion source temperature (TEM): 450^0C; Ion source gas 1 (GS1): 36 psi; Ion source gas 2 (GS2): 45 psi; Collision gas (CAD): medium; Entrance potential (EP): 10 V; Collision cell exit potential (CXP): 10 V; Interface heater (Ihe), on. Quadrupole 1 and quadrupole 3 were maintained at unit resolution. Dwell time set was 100 ms for all compounds.

Table 1

Mass spectrometry parameters.

Analyte	RT (m/z)	MRM (m/z)	CE (m/z)	DP (m/z)
PPD	1.6	109→92*	22	45
		109→65	33	45
MAPPD	2.0	151→133	20	28
		151→109	28	28
		151→92*	32	28
DAPPD	5.1	193→151	25	60
		193→109	37	60
		193→92*	42	60
Acetanilide (IS)	6.1	136→94	21	45
		136→77	36	45

* Transition used in the quantification.

2.5. Calibrators and controls

Stock solution of PPD with a concentration of 1.0 mg/mL was prepared by dissolving 10 mg free base of PPD-dihydrochloride in 10 mL water. Stock solutions of MAPPD and DAPPD (1 mg/mL) were prepared separately by dissolving 10 mg of each analyte in 10 mL methanol. Working solutions were prepared by diluting the stock solutions of each analyte to a final concentration of 100 µg/mL. Different stock standards were used to prepare quality control (QC) samples at the same concentrations. Working calibrators (10, 50, 100, 500, 1000 and 2000 ng/mL) for PPD, MAPPD and DAPPD were made in blank blood. Low, medium and high quality controls (LQC, MQC and HQC) were also prepared in blank blood at concentration of 75, 750 and 1500 ng/mL for all analytes. Working internal standard containing 200 ng/mL of acetanilide was prepared by diluting the stock solutions of acetanilide (1.0 mg/mL) with methanol. Standard solutions were stored at -20 ^{0}C until use.

2.6. Sample preparation

To 10 mL polypropylene tubes was added; 0.5 mL of blood, 100 µL of 200 ng/mL acetanilide (IS), 100 µL of concentrated ammonium hydroxide (33%) and 4.0 mL of dichloromethane. The tubes were then vortex mixed for 5-min and centrifuged for 3 min. The organic layer was transferred to 7 mL glass tubes and evaporated to dryness using a speed vacuum concentrator at 35^{0}C. The dried extracts were reconstituted in 100 µL of 1% formic acid in acetonitrile and 10 µL was injected into the LC-MS/MS system.

2.7. Validation

The method was validated to general requirements to meet the USFDA guidelines [19]. Specificity, sensitivity, linearity, inter- and inter-assay imprecision, accuracy, recovery, matrix effect, dilution integrity and stability were assessed to evaluate method integrity.

2.7.1. Specificity, sensitivity and linearity

Seven different blank blood specimens (no analyte or IS) were used to evaluate the co-eluting chromatographic peaks that might interfere with detection of analytes or IS.

The limits of detection (LOD) and quantification (LOQ) for each analyte were determined as analyte concentrations giving signal-to-noise ratio (S/N) of 3 and 10, respectively.

Calibration graphs for PPD, MAPPD and DAPPD were established in the range of 10–2000 ng/mL blood. Calibration curves were constructed by plotting the peak area ratio of the analyte to the IS versus analyte concentration. Linearity of the method based on peak area ratios was evaluated by coefficient of determination (r^2).

2.7.2. Imprecision, accuracy and dilution integrity

Intra- and inter-assay accuracy and precision for each analyte were estimated at HQC, MQC and LQC (75, 750 and 1500 ng/mL) in five replicates. The accuracy and imprecision was calculated and expressed in terms of percent bias and percent relative standard deviation (%RSD), respectively.

To investigate dilution integrity, spiked sample at concentration of 10000 ng/mL was prepared and diluted 10 times with blank blood in four replicates. Each concentration was calculated by applying the dilution factor of 10 against the freshly prepared calibration curve for PPD, MAPPD and DAPPD.

2.7.3. Matrix effects, recovery and stability

To evaluate the magnitude of matrix ion suppression/ enhancement effects on the MRM LC–MS/MS sensitivity, a comparison between the peak areas of extracted blank blood samples spiked with standards at QC2 concentrations after the extraction procedure and the peak areas of pure diluted standards at the same concentrations [20].

The extraction efficiency (%) of the analytes was determined at HQC, MQC and LQC (n = 4). It was calculated by comparing peak areas obtained from the extracted samples to with those achieved after direct injections of standard solutions at the same concentrations.

In order to assess the stability of the analytes in human blood, two quality control samples MQC and LQC (n = 4) were prepared and stored at -20 ^{0}C for 6, 12, 24 and 48 h. Concentrations were calculated and the corresponding means were compared.

Stability of analytes after extraction was evaluated over 24 h. Extracted QCs were analyzed immediately after extraction along with calibrators, and re-injected again after 4, 6, 12 and 24 h.

2.8. Application of the method

Antemortem and *postmortem* blood samples were collected from poisoning cases by PPD at Assiut Chemical Laboratory, Medico-Legal Department, Ministry of Justice, and Hospital Sohag University, Egypt. This study protocol was approved by the ethics advise committee of Medico-Legal Department, Ministry of Justice. Postmortem human blood samples were stored at -20 ^{0}C until analysis.

3. Results and discussion

3.1. Optimization of the chromatographic conditions and extraction

Various mixture(s) of isocratic of acetonitrile or methanol with different buffers such as ammonium acetate, ammonium formate, acetic acid and formic acid at variable pH range of 3.5–5.5 were tested for complete chromatographic resolution of PPD, MAPPD, DAPPD, and IS.

Also, different gradients of acetonitrile and 0.1% formic acid were assayed at a constant flow rate of 1.0 mL/min in order to get all analytes separated. Mobile phase comprising of 0.1% formic acid:acetonitrile in gradient pumped at 1 mL/min was found to be suitable during LC optimization. The retention times of the PPD, MAPPD, DAPPD and the internal standard (IS; acetanilide) were 1.64, 2.01, 5.09 and 6.13 min, respectively

Since PPD is a water-soluble, it was necessary in the method development to optimize extraction procedure. In this work, one-step liquid–liquid or solid phase extraction were tested to isolate PPD, MAPPD and DAPPD from blood samples. Liquid‑liquid extraction was examined using different organic solvent such as dichloromethane, ethylacetate, methyl-tert-butyl and ethylacetate:hexane (1:4). A clean chromatogram and sufficient extraction recoveries for all analytes were obtained with dichloromethane. A different solid phase extraction cartridge such as Bond elut Plexa, C18 and C8 cartridges has been tested. Very poor recoveries were obtained for both PPD and MAPPD (15-20 %) with all cartridges.

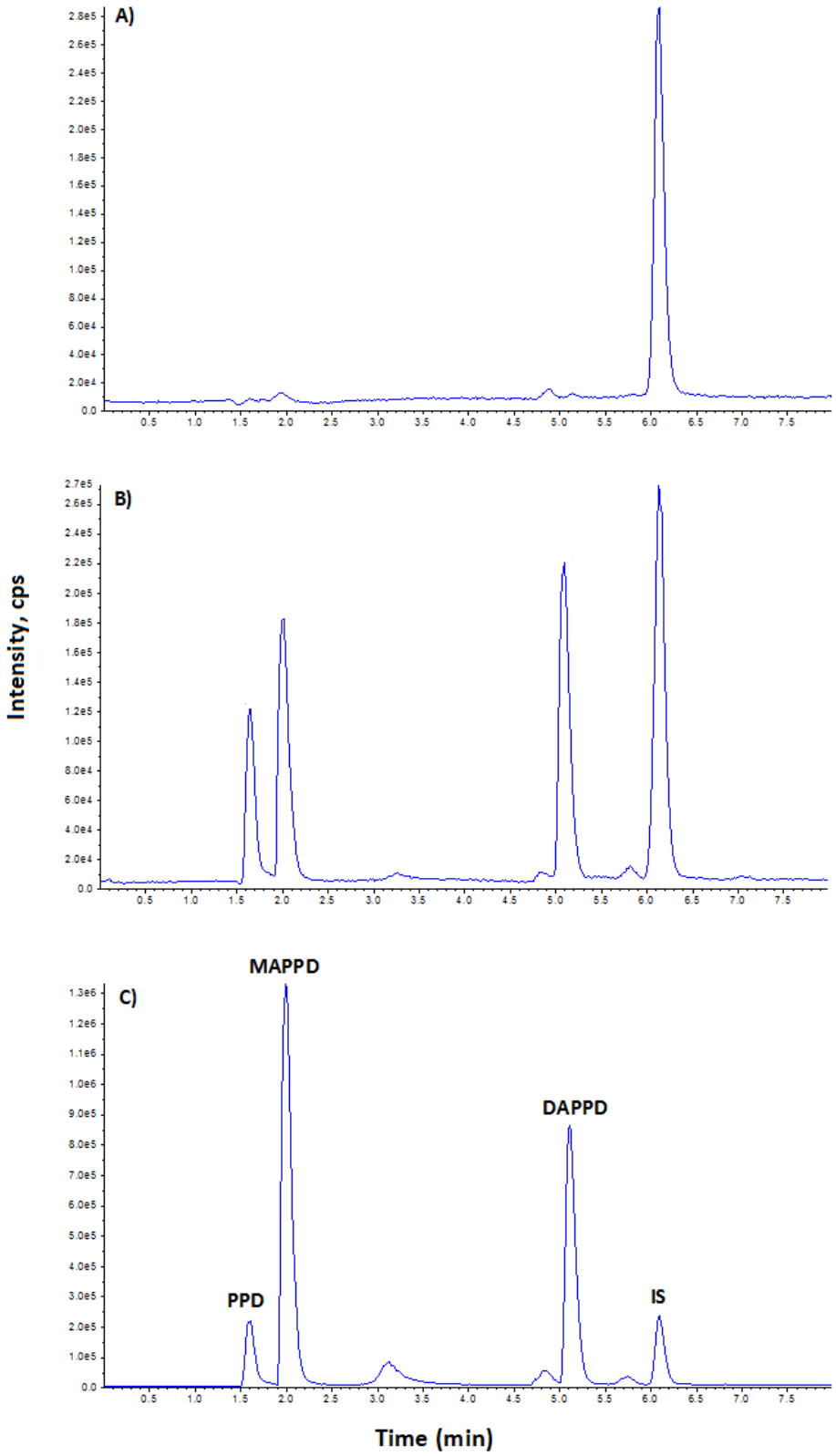

Fig 2. Total ion chromatograms (TIC) for the analysis of a blank blood with IS (A), blood sample spiked with 30 ng/mL of PPD, MAPPD and DAPPD (B) and blood sample collected from *postmortem* case (C).

Table 2
Summary of calibration curves for PPD, MAPPD and DAPPD in human blood (n = 3) with back calculated concentrations.

	Concentration (ng/mL)						Regression Equation	
	10	50	100	500	1000	2000	Slope	r^2
PPD								
Mean	10.6	53.9	108.3	519.3	971.7	1960.0	0.0140	0.9853
SD	0.3	3.8	4.0	35.5	59.7	113.6	0.0069	0.0127
% RSD	2.9	7.1	3.7	6.8	6.1	5.8	0.4929	0.0129
%Bias	5.7	7.9	8.3	3.9	-2.8	-2.0		
MAPPD								
Mean	10.3	49.0	109.3	522.7	1008.3	2086.7	0.0196	0.9807
SD	0.6	1.8	5.0	32.1	28.4	181.5	0.0060	0.0083
% RSD	5.5	3.7	4.6	6.1	2.8	8.7	0.3049	0.0084
% Bias	2.7	-1.9	9.3	4.5	0.8	4.3		
DAPPD								
Mean	11.1	48.7	110.0	520.7	1022.0	2060.0	0.0099	0.9897
SD	0.4	4.1	6.1	26.1	87.0	120.0	0.0021	0.0154
% RSD	3.9	8.4	5.5	5.0	8.5	5.8	0.2073	0.0155
% Bias	11.0	-2.6	10.0	4.1	2.2	3.0		

SD: Standard deviation
RSD: Relative standard deviation
r^2: Correlation coefficient

3.2. Method validation

3.2.1. Specificity, sensitivity and linearity

Six different blank blood specimens were analyzed to evaluate chromatographic interference. Figure 2$_{A \text{ and } B}$ represent the total ion chromatogram (TIC) for a blank blood fortified with internal standard, and fortified with LQC. As shown in these figures, no interferences with analytes peaks were detected.

The LOD and LOQ for all analytes were 5 and 10 ng/mL, respectively.

The calibration lines for PPD, MAPPD and DAPPD showed good linearity over the dynamic range 10–2000 ng/mL within three regression curves. Calculated concentrations of each calibrator were compared to target and were within ±20%. An overview of characteristic calibration data over a dynamic range from the LOD/LOQs to 2000 ng/mL for PPD, MAPPD and DAPPD is presented in Table 2.

3.2.2. Precision and accuracy and dilution integrity

Precision and accuracy of the method are presented in Table 3. Intra- and inter-assay imprecisions for all analytes were ranged from 3.67 to 11.18% and 5.58 to 13.27% (RSD), respectively. Inter- and inter-assay assay accuracy for all analytes ranged from -14.00 to 5.17% and -9.47 to 0.86% (% bias), respectively.

Dilution accuracy was studied by assaying diluted blood quality control samples. The accuracy for 1/10 dilution samples for all compounds was ranged from -14.00 to -0.44%.

3.2.3. Matrix effect and recovery

With regard to the matrix effect, the comparison between relative standard deviation of the analytes spiked in extracted blank blood samples versus those for pure diluted standards showed less than 10% analytical signal suppression.

Mean recovery values for PPD, MAPPD and DAPPD were 51.94, 56.20 and 54.88%, respectively.

3.2.3. Stability

Analyte stability evaluations are presented in Table 4. PPD was reduced to 85% of the original value during the first 6 h and to 3.1% after 24 h. The Critical degradation of PPD may be due to

Table 3
Accuracy and imprecision for the simultaneous determination of PPD, MAPPD and DAPPD in human blood

Analyte (ng/mL)	Intra-assay (n = 6)			Inter-assay (n = 18)		
	Mean (ng/mL)	Accuracy (%Bias)	Precision (% RSD)	Mean (ng/mL)	Accuracy (% Bias)	Precision (% RSD)
PPD						
75	68.02	-9.31	3.67	69.09	-7.88	10.30
750	757.50	1.00	7.93	725.59	-3.253	13.27
1500	1290.00	-14.00	6.58	1385.00	-7.67	13.25
MAPPD						
75	65.01	-13.32	11.18	69.71	-7.06	12.39
750	688.33	-8.22	5.49	734.47	-2.07	9.89
1500	1398.00	-6.80	5.62	1358.00	-9.47	5.58
DAPPD						
75	78.88	5.17	7.60	75.65	0.86	7.77
750	715.75	-4.57	5.95	706.88	-5.75	9.03
1500	1498.00	-0.13	3.96	1427.75	-4.82	12.72

Table 4
Stability data of PPD, MAPPD and DAPPD in human blood at time interval (n = 4).

Analyte	Target (ng/mL)	Stability in blood[a] Mean concentration (% of initial concentration)[b]					Extracts stability Mean concentration (% of initial concentration)[b]				
		0 h	6 h	12 h	24 h	48 h	0 h	4 h	6 h	12 h	24 h
PPD	75	100.0 (12.5)	87.7 (4.6)	38.2 (13.2)	22.1 (11.6)	5.5 (10.5)	100.0 (12.5)	98.1 (11.3)	97.5 (4.8)	41.3 (8.2)	3.1 (12.8)
	750	100.0 (16.6)	85.0 (12.8)	29.2 (14.9)	27.5 (8.7)	11.7 (14.7)	100.0 7.5)	95.6 (13.4)	90.1 (10.9)	76.0 (11.8)	17.4 (10.3)
MAPPD	75	100.0 (12.9)	90.3 (14.0)	87.2 (11.3)	86.6 (1.4)	83.3 (11.7)	100.0 (13.3)	98.8 (11.9)	103.0 (8.2)	86.0 (12.3)	87.0 (14.7)
	750	100.0 (8.6)	102.7 (4.7)	98.3 (9.0)	97.5 (12.6)	94.1 (13.9)	100.0 (5.9)	99.2 (4.7)	98.6 (4.3)	93.9 (15.1)	90.2 (14.2)
DMPPD	75	100.0 (6.9)	108.5 (4.0)	111.4 (4.4)	99.0 (15.0)	98.1 (9.4)	100.0 (10.0)	111.2 (6.3)	103.9 (4.0)	105.1 (12.2)	110.8 (1.8)
	750	100.0 (13.7)	112.6 (6.6)	114.3 (13.9)	101.5 (6.4)	100.8 (14.2)	100.0 (9.4)	112.6 (6.6)	106.3 (3.7)	113.9 (6.8)	115.9 (9.1)

[a] Stability at -20 ^{0}C
[b] Mean (RSD)

the autoxidation of PPD by air and/or light [21-22]. Ascorbic acid was used as antioxidant to prevent the oxidation of PPD [18]. In this work, the ascorbic acid was tested to prevent the oxidation of PPD; a significant degradation of PPD was also resulted. PPD was observed to be stable in strong acidic medium or as a hydrochloride salt. To minimize the oxidation of PPD, the samples were extracted in short time and stored in brown vials before the analysis. MAPPD and DAPPD were stable in blood samples and the autosampler up to 48 h and 24 h, respectively. The RSD for the two quality control samples of MAPPD and DAPPD was within 15%.

3.3. Application of the method

The validated method was successfully applied to quantify PPD, MAPPD and DAPPD from poisoning cases by PPD. Figure 2 shows the Total Ion Chromatogram (TIC) of a blood sample spiked with PPD, MAPPD and DAPPD and *postmortem* blood collected from autopsy cases with suspected PPD poisoning. The concentrations of PPD, MAPPD and DAPPD in antemortem and postmortem blood samples are presented in Table 5.

Table 5

Concentration levels of PPD, MAPPD and DAPPD (µg/mL) for 8 post-mortem and one antemortem cases.

Cases	PPD	MAPPD	DAPPD
1	3.408	3.987	1.767
2	0.631	2.703	1.000
3	0.287	3.015	15.076
4	0.065	2.636	14.946
5	12.503	4.802	1.879
6	5.876	5.316	2.140
7	0.287	6.913	11.425
8	0.015	0.828	5.119
9*	0.003	0.235	2.354

* Antemortem case

4. Conclusion

To our knowledge, this is the first fully validated LC–MS–MS method for the simultaneous identification and quantification of the PPD and its metabolites MAPPD and DAPPD in human blood. The method was successfully applied to analysis of blood samples collected from poisoning cases by PPD.

Acknowledgments

This research was supported by the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC). The authors would like to acknowledge the Department of Pharmacology, University of Pretoria and Assiut Chemical Laboratory of Egyptian Medico-Legal Department for providing necessary facilities to carry out this work. We would also like to thank all staff of Clinical Toxicology Laboratory of Faculty of Medicine, Sohag University, Egypt for providing biological samples.

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