Application of a LC-MS/MS method developed for the determination of *p*-phenylenediamine, *N*-acetyl-*p*-phenylenediamine and *N,N*-diacetyl-*p*-phenylenediamine in human urine specimens

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ABSTRACT

Cases of poisoning by *p*-phenylenediamine (PPD) are detected sporadically. Recently an article on the development and validation of a LC-MS/MS method for the detection of PPD and its metabolites, *N*-acetyl-*p*-phenylenediamine (MAPPD) and *N,N*-diacetyl-*p*-phenylenediamine (DAPPD) in blood was published. In the current study this method for detection of these compounds was validated and applied to urine samples. The analytes were extracted from urine samples with methylene chloride and ammonium hydroxide as alkaline medium. Detection was performed by LC-MS/MS using electrospray positive ionization under multiple reaction-monitoring mode. Calibration curves were linear in the range 5–2000 ng/mL for all analytes. Intra- and inter-assay imprecisions were within 1.58–9.52% and 5.43–9.45% respectively, for PPD, MAPPD and DAPPD. Inter-assay accuracies were within -7.43 and 7.36 for all compounds. Lower limit of quantification was 5 ng/mL for all analytes. The method, which complies with the validation criteria, was successfully applied to the analysis of PPD, MAPPD and DAPPD in human urine samples collected from clinical and postmortem cases.

Keywords: *p*-Phenylenediamine; Urine; LC-MS/MS; Validation; Application

Introduction

p-Phenylenediamine (PPD) is widely used in the manufacturing industry and as a hair and skin dye in combination with Henna (Ashraf *et al.*, 1994; Chugh *et al.*, 1982; Zeggwagh *et al.*, 2003). The number of poisoning cases due to PPD appears to be increasing (Chrispal *et al.*, 2010; Kumar, 2010; Shalaby et al., 2010; Mohamed *et al.*, 2014).

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 and excreted in urine (Nakao and Takeda, 1979; Goetz *et al.*, 1988; Kawakubo *et al.*, 2000)

Gas chromatography–mass spectrometry (GC–MS) (Stambouli, *et al.*, 2010), high performance liquid chromatography (HPLC) (Wang and Tsai, 2003; Meyer *et al.*, 2009; Hooff *et al.*, 2011) and MALDI-MS/MS (Hooff *et al.*, 2011) has been used for the detection of PPD and its metabolites in biological samples. Recently an LC-MS/MS method was published for the detection of PPD and its metabolites, MAPPD and DAPPD in blood samples collected at post-mortem (Mohamed, *et al.*, 2015). In this study the method was validated and applied to determine PPD and its metabolites in urine samples collected from clinical and postmortem cases.

Materials and methods

Reagents and standards

p-Phenylenediamine dihydrochloride (99%), MAPPD (99%), DAPPD and acetanilide (99%) were purchased from Sigma-Aldrich. Formic acid (98%) was purchased from Fluka Chemie (GmbH). Acetonitrile (99.9%) and ammonium hydroxide (33%) were purchased from Merck. Methylene chloride was purchased from Baxter. Methanol (99.9%) was purchased from Romil pure Chemistry.

LC–MS/MS methodology

The analyses were carried out on an Agilent LC 1100 binary pump, autosampler, vacuum degasser, and column oven (Agilent Technologies). Separation was

accomplished on an Eclipse XDB C18 column (150 mm x 4.6 mm, 5μ), using acetonitrile (solvent A) and 0.1% formic acid (solvent B) in gradient elution at a flow rate of 1.0 mL/min. 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. The column temperature was held at 25°C and the injection volume was 10.0 µL. Total run time was 8 min. 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.

Detection of analytes and IS was performed on a triple quadrupole mass spectrometer (AB/MDS Sciex 4000 QTrap LC–MS/MS instrument; Applied Biosystems, Canada) operating in the positive mode (ESI^+). The product ion scanning spectra of the three target compounds and IS, collision energy and declustering potential were set as reported earlier (Mohamed, *et al.*, 2015). Quantitation was done using multiple reaction monitoring (MRM) mode to monitor protonated precursor→product ion transition of m/z 109→92 for PPD, m/z 151→92 for MAPPD, m/z 193→92 for DAPPD and m/z 136→77 for IS.

Validation

Method integrity was evaluated to the requirements of the USFDA guidelines (FDA, 2011). Calibrators and controls were prepared as determined previously (Mohamed, *et al.*, 2015). Extraction of the target analytes and IS from urine (0.5 mL) involved LLE using methylene chloride and ammonium hydroxide as described for blood samples (Mohamed, *et al.*, 2015).

For method *specificity*, seven different blank urine specimens were evaluated for co-eluting chromatographic peaks that might interfere with detection of the analytes or IS. *Sensitivity* of the method was evaluated by determining limits of detection (LOD) and quantification (LOQ) for each compound. LOD was defined as the lowest concentration for which analyte ion signal-to-noise ratio (S/N) was ≥3 (determined by peak height). LOQ was defined as lowest concentration for which analyte ion signal-to-noise ratio was ≥10 and had a measured concentration within ±20% of target in five replicates. *Linearity* of the method was investigated by evaluation of the regression line and expressed as coefficient of determination (r^2). Linearity was

achieved with a minimal r^2 of 0.99. Calibration curves (n = 3) were prepared by spiking PPD, MAPPD and DAPPD stock solutions with blank urine to obtain calibration concentrations within 5–2000 ng/mL. Calibration curves were constructed by plotting the peak area ratio of the analyte to the IS versus analyte concentration.

Intra- and inter-assay *accuracy and imprecision* for PPD, MAPPD and DAPPD were determined by 5 replicate analyses of 3 different concentrations: HQC, MQC and LQC (75, 750 and 1500 ng/mL). Intra-assay data were assessed by comparing data from within one run (n = 5) and inter-assay data were determined between three separate runs (n = 15). The accuracy and imprecision was calculated and expressed in terms of percent bias and percent relative standard deviation (%RSD), respectively.

Stability of the analyte in urine was tested at LQC and MQC in samples (n = 4) stored for 36 h at room temperature, in the fridge (4°C) and when kept in a freezer (-20°C). For evaluation of *matrix effect*, the peak areas of extracted drug-free urine samples spiked with standard solution after the extraction procedure were compared to the peak areas of the standard solution at the same concentrations (Matuszewski, *et al.*, 2003). Analytical *recoveries* were calculated by comparing the peak areas obtained when QC samples were analyzed by adding the analytical reference standards and the IS in the extract of drug-free urine samples prior to and after the extraction procedure. The recoveries were assessed by QC samples using four replicates for each concentration level.

Dilution integrity was investigated by diluting quality control sample (100 µg/mL) with blank urine. Four replicate samples of 1/100 dilution were prepared and their concentrations were calculated by applying the dilution factor of 100-fold against the freshly prepared calibration curve for PPD, MAPPD and DAPPD.

Application of the method

Human urine samples were received from the Medico-Legal Department, Ministry of Justice and Sohag Hospital University, Egypt. All patients were diagnosed with clinical symptoms of intoxication by PPD. The need for a written consent by the Ethical committee was waived, due to the fact that samples were collected on a routine basis and no study was designed prior to sample collection. The time interval between urine collection and storage in the freezer at -20^{0}C was < 2 h.

Results

Method validation

Representative MRM chromatograms for blank urine without internal standard, and fortified with LOQ, are presented in Figure $1_{A \text{ and } B}$. No interferences with analyte peaks were detected in the blank specimens analyzed. LOD and LOQ for all analytes were 2.5 and 5 ng/mL, respectively.

The calibration curves (n=3) for PPD, MAPPD and DAPPD were linear over the dynamic range 5–2000 ng/mL within three regression curves. Calculated concentrations of each calibrator were compared to the target and were within ±10%. The calibration data for PPD, MAPPD and DAPPD is summarized in Table I.

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

	Concentration (ng/mL)						Regression Equation	
	5	50	100	500	1000	2000	Slope	r^2
PPD								
Mean	4.6	47.8	91.5	456.8	973.6	1921.3	0.0104	0.9985
SD	0.2	2.0	3.5	23.9	89.0	103.6	0.0059	0.0015
% RSD	4.2%	4.1%	3.8%	5.2%	9.1%	5.4	0.5629	0.0015
%Bias	-8.4%	-4.4%	-8.5%	-8.6%	-2.6%	-3.9%		
MAPPD							0.0072	0.9978
Mean	5.0	51.6	106.1	509.7	993.8	1959.6	0.0059	0.0018
SD	0.2	4.1	3.8	29.1	63.9	131.0	0.8171	0.0018
% RSD	3.0	7.9	3.6	5.7	6.4	6.7		
% Bias	-0.9	3.3	6.1	1.9	-0.6	-2.0		
DAPPD								
Mean	4.8	53.8	102.1	539.9	1036.4	1912.8	0.0079	0.9986
SD	0.3	2.9	7.9	28.6	37.9	75.2	0.0036	0.0019
% RSD	6.4	5.4	7.8	5.3	3.7	3.9	0.4590	0.0019
% Bias	-4.7	7.6	2.1	8.0	3.6	-4.4		

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

Imprecision and accuracy of the method were evaluated at three concentrations (LQC, MQC, HQC) over the linear dynamic range and are presented in Table II. Five replicates at each concentration were assayed to determine intra-assay accuracy and precision. Intra- and inter-assay imprecisions for all analytes were within 1.58–9.52% and 5.43–9.45% (RSD), respectively. Inter-assay accuracy for PPD, MAPPD and DAPPD ranged between -7.43 and 7.36 (% bias).

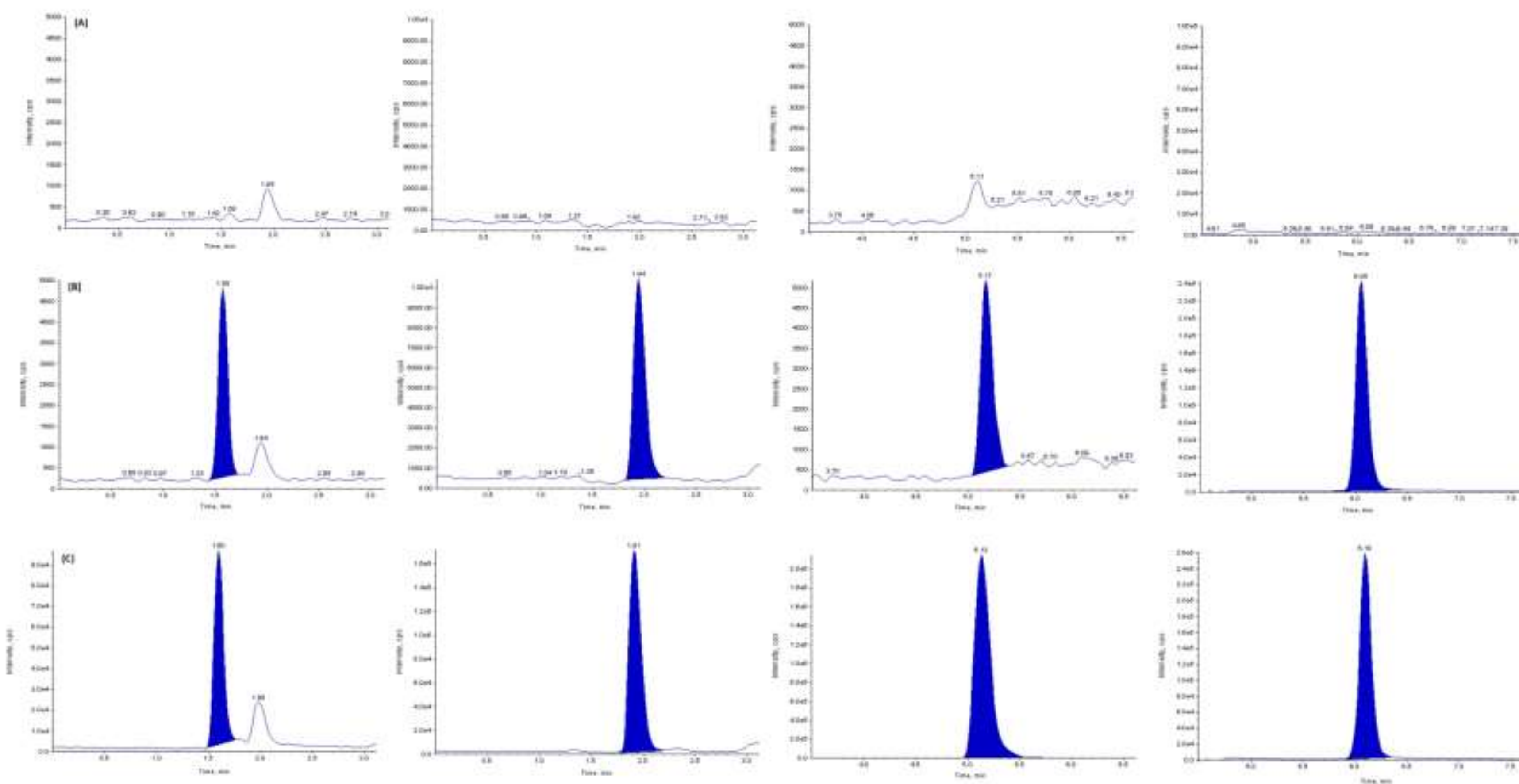

Fig 1. MRM chromatograms for PPD (*m/z* 109 → 92), MAPPD (*m/z* 151 → 92), DAPPD (*m/z* 193 → 92) and IS (*m/z* 136 → 77) after the analysis of A) double bank urine, B) LOQ (5 ng/mL) and C) authentic urine sample after 1:50 dilution with blank urine, the concentrations of PPD, MAPPD and DAPPD were 0.42, 0.35 and 9.08 ug/mL.

Table II: Accuracy and imprecision for PPD, MAPPD and DAPPD in human urine.

Analyte (ng/mL)	Intra-assay (n = 6)			Inter-assay (n = 15)		
	Mean (ng/mL)	Accuracy (%Bias)	Imprecision (% RSD)	Mean (ng/mL)	Accuracy (% Bias)	Imprecision (% RSD)
PPD						
75	79.63	6.18	7.62	80.52	7.36	5.43
750	793.00	5.73	8.62	792.55	5.67	7.80
1500	1323.33	-11.83	8.08	1472.86	-1.81	8.10
MAPPD						
75	83.00	10.67	1.58	76.93	2.57	7.14
750	727.50	-3.00	7.06	749.13	-0.12	6.95
1500	1340.00	-10.67	5.48	1393.33	-7.11	6.91
DAPPD						
75	69.88	-6.83	5.05	69.43	-7.43	6.39
750	705.33	-5.96	9.52	770.78	2.77	8.03
1500	1305.00	-13.00	2.38	1472.31	-1.85	9.45

From the stability experiments it was evident that PPD in urine stored for 36 h at room temperature and 4^0C was reduced to 72.44 and 75.56%, respectively, whereas when frozen (-20°C), PPD remained stable. The stability data indicated that PPD has a higher degree of stability in urine compared to blood (Mohamed, *et al.*, 2015). The latter may be attributed to the acidity of urine as PPD has been found to have better stability in acidic storage conditions (Hooff *et al.*, 2011; Mohamed, *et al.*, 2015). MAPPD and DAPPD were stable under all conditions. Stability results are provided in Table III.

Table III: Stability of PPD, MAPPD and DAPPD in human urine after 36 hours (*n* = 4).

Analyte	QC sample (ng/mL)	Concentration of analyte (ng/mL)		
		R.T. Mean (%RSD)	4 ^{0}C Mean (%RSD)	- 20 ^{0}C Mean (%RSD)
PPD	75	54.33 (3.83)	56.67 (5.39)	80.00 (6.25)
	750	496.67(3.08)	529.00 (6.71)	826.67 (4.39)
MAPPD	75	69.30 (6.69)	66.67 (1.74)	71.47 (8.63)
	750	779.00 (2.58)	754.00 (2.42)	760.67 (9.36)
DAPPD	75	70.90 (5.31)	66.33 (4.81)	77.70 (3.29)
	750	766.67 (4.54)	779.33 (6.30)	742.33 (9.68)

QC Quality Control
R.T. = Room Temperature

Table IV summarizes the mean values of recovery, matrix effect and corresponding %RSD. Mean recovery values for PPD, MAPPD and DAPPD were 57.62%, 74.19% and 50.99%, respectively. The method showed satisfactory recoveries for most of the analytes. The matrix effect of the method was estimated by the comparison between peak areas of analytes spiked in extracted blank urine samples versus those for pure diluted standards which showed less than 15% analytical signal enhancement due to co-eluting endogenous substances.

Table IV: Extraction recovery and matrix effect of PPD, MAPPD and DAPPD in human urine.

Analyte	QC sample (ng/mL)	Recovery		Matrix effect*	
		(%)	%RSD	(%)	%RSD
PPD	75	51.47	1.53		
	750	55.30	7.73	110.86	12.72
	1500	66.10	13.67		
MAPPD	75	82.64	9.93		
	750	72.68	8.06	114.29	5.28
	1500	67.24	5.08		
DAPPD	75	47.30	1.83		
	750	51.07	2.63	112.47	3.98
	1500	54.61	2.65		

QC = Quality Control
* MQC (750 ng/mL) used from matrix effect evaluation

Dilution accuracy was studied by assaying diluted urine quality control samples. The accuracy for 1/100 dilution samples for all compounds ranged between 3.75% and 8.55%.

Application of the method

The method was successfully applied to detect and quantify PPD, MAPPD and DAPPD in human urine samples in ten poisoning cases. The precision and accuracy for calibration and QC samples were well within the acceptable limits. High concentration levels of PPD, MAPPD or DAPPD in urine samples were diluted by blank urine to fall in the range of calibration curve. In Figure 1_C the MRM chromatograms for the analysis of PPD, MAPPD and DAPPD in the urine sample of a patient with suspected PPD intoxication is presented. The concentrations of PPD, MAPPD and DAPPD in the patient samples are presented in Table V.

Table V: Concentration levels of PPD, MAPPD and DAPPD (µg/mL) in urine for clinical (suicidal and accidental) and postmortem cases.

Cases	PPD	MAPPD	DAPPD
1	64.79	296.70	74.91*
2	78.66	103.52	62.31*
3	8.24	180.37	73.82*
4	0.42	0.35	9.08
5	0.09	0.40	2.02
6	0.18	0.44	0.28
7	0.03	1.95	2.88
8	0.02	0.12	0.15
9	0.09	0.40	2.02
10	0.18	0.44	0.28

* Postmortem cases

Discussion

A validated method providing specific and accurate results for the simultaneous quantification of PPD, MAPPD and DAPPD in human urine by LC–MS/MS with ESI^+ and liquid–liquid extraction was described. This was achieved over an analyte concentration range that is consistent with expected PPD urine concentrations providing potential use as an analytical procedure in the fields of clinical and forensic toxicology.

High sensitivity is a continuous goal, LOQ for PPD, MAPPD and DMPPD in blood and urine samples was 10 ng/mL and 5.4 µg/mL (50 µmol/L), respectively for all analytes (Hooff *et al.*, 2011; Mohamed, *et al.*, 2015). The present validated method provides a good sensitivity with LOQ of 5 ng/mL for all analytes.

The concentrations of PPD, MAPPD and DAPPD in the patients' samples contain individual variation as expected (Table V). The individual variations are ascribed to the discrepancy of metabolic capacity, individual constitution, underlying disease and the degree of diseases as well as the amount ingested. The liver and kidney are the main organs responsible for biotransformation and elimination of drugs and their metabolites from the body (Verbeeck and Musuamba, 2009). Factors affecting renal excretion of drugs, causing variability between patients include; kidney function, plasma drug concentration, plasma binding protein, urine pH and urine flow (Regardh, 1985). Age is also reported to affect renal clearance. The latter being reduced in neonates and the elderly. Disease states that impair elimination of drugs

are diabetes and hypertension (Verbeeck and Musuamba, 2009). It is not known whether any of the patients had either of these diseases. Furthermore, drug metabolizing enzymes and transporters are affected in patients with renal disease, which could result in excessive accumulation and drug toxicity (Verbeeck and Musuamba, 2009). Drug transporters such as P-glycoprotein mediate the excretion of many drugs (Lee and Kim, 2004), and if any of the patients had renal disease this may explain the variability in elimination of PPD and its metabolites.

The results indicate that the concentrations of PPD and its metabolites were much higher in the postmortem samples than in the clinical samples (Table 5). Although the reasons for these differences between clinical and post mortem samples still need to be ascertained, there are some possible explanations. It is known that drugs can be altered in the postmortem environment owing to pH changes, which change the ionization of the drug, causing it to undergo chemical changes (Ferner, 2008). For the clinical samples, PPD and its metabolites would have remained stable as these compounds are known to be more stable in an acidic environment (Hooff *et al*., 2011). In postmortem cases the pH of the urine was found to be higher than that of clinical cases and could have led to chemical changes taking place; hence the big difference in concentrations between clinical and postmortem cases.

Redistribution in the case of postmortem samples would not explain the difference in concentrations detected in postmortem vs. clinical samples as PPD and metabolites are excreted in urine within 24 h. Clinical patients usually present to hospital only 12 h or longer after acute ingestion (Hooff *et al*., 2011), at which point a substantial amount of the toxin has been voided in urine. Postmortem reports suggest that, when poisoning was fatal, patients died within 6 h of ingesting PPD, providing less time for voiding of urine. Furthermore, the difference in concentrations of PPD and its metabolites may be explained by the concentration of the toxin ingested/absorbed and the route of administration. In postmortem cases PPD was taken orally at higher concentrations for intentional poisoning. In contrast, clinical cases of accidental poisoning occurred via absorption through the skin resulting in relatively low systemic concentrations.

In conclusion, the validated method is considered sensitive enough for analysis of PPD and its metabolites in urine samples. Addidional patient information, which could not be obtained, would contribute to a better understanding of the severity of intoxication, and therefore the correlation with PPD, MAPPD and DAPPD levels.

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ABBREVIATIONS USED

DAPPD

N,*N*-diacetyl-*p*-phenylenediamine

MAPPD

N-acetyl-*p*-phenylenediamine

PPD

p-phenylenediamine.

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