Journal of Chromatography B, 997 (2015) 1–6

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Development and validation of an LC–MS/MS method for determination of *p*-phenylenediamine and its metabolites in blood samples

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ARTICLE INFO

Article history:
Received 1 November 2014
Received in revised form 11 March 2015
Accepted 25 May 2015
Available online 28 May 2015

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

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.

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 the manufacturing 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 number 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 edema of the epiglottis and vocal folds were observed in all cases [8]. 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 the 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 develop 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 post-mortem cases provided by Assiut Forensic Chemistry Laboratory of Medico-Legal Department, Ministry of Justice, Egypt.

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http://dx.doi.org/10.1016/j.jchromb.2015.05.030
1570-0232/

Table 1
Mass spectrometry parameters.

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

[a] Transition used in the quantification.

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 an Eclipse XDB C18 column (150 mm × 4.6 mm, 5 μ) were used 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 °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 10 μL/min. For each compound, two or three mass 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 de-clustering 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 °C; 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.

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 °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 °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 a concentration of 10,000 ng/mL was prepared and diluted 10 times with