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Article

Development and Validation of UPLC-Qtof-MS Method for Blood Analysis of Amphetamine-Related Drug Isomers

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Abstract: Abuse of amphetamine-related drugs (ARDs) causes traffic accidents, violence, and overdose. In forensic toxicology, analysis for ARDs in biological samples can help identify those driving or performing other tasks under the influence of drugs, clarify the cause of death, and identify recent drug users. In this study, we validated a pseudo-isocratic UPLC-qTOF-MS method following mixed mode cation exchange (MMSPE) extraction for analysis of ARDs in blood. The procedure requires 250 µL of blood to achieve a limit of quantification (LOQ) and detection (LOD) of 20 ng/mL for all analytes. In aged animal blood samples, extraction recoveries of 63-90% and matrix effects of 9-21% were observed. Precision and accuracy for all analytes were within 20% and 89–118%, respectively. The analytical method was developed and validated in accordance with the Scientific Working Group for Forensic Toxicology (SWGTOX) Standard. It has acceptable accuracy and precision for use in doping control and forensic toxicology.

Keywords: Amphetamine-related drugs; Forensic Toxicology; blood; UPLC-qTOF-MS; MMSPE; Validation; SWGTOX

1. Introduction

Amphetamine-related drugs (ADRs) are stimulant drugs that possess similar structure and functions of endogenous amines, such as dopamine [1]. These sympathomimetic drugs are composed of a phenyl ring connected to an amine group through a two-carbon side chain bearing a methyl group. ARDs include a wide range of compounds, such as methamphetamine (MAMP), beta-methylphenethylamine (BMPEA), ephedrine (EPH), pseudoephedrine (PEPH), norephedrine (NEPH), norpseudoephedrine or cathine (CAT), methylenedioxyamphetamine (MDA), methylenedioxymethamphetamine (MDMA), methylenedioxyethylamphetamine (MDEA), and phentermine (PHE). These compounds release catecholamines, such as dopamine, from the presynaptic cleft at both the central and peripheral sites [1].

ARDs are a distinct class of sympathomimetic compounds. Based on their origin, they can be categorized into synthetic (AMP, BMPEA, MAMP, MDA, MDEA) and naturally-occurring ARDs (EPH, PEPH, NEPH, and CAT) [2]. In this study, a mixture of synthetic and naturally-occurring ARDs, such as AMP, BMPEA, CAT, EPH, NEPH, and PEPH, has been chosen based on their epidemiological abuse in Saudi Arabia. Structurally, two pairs of these analytes are diastereomers (EPH and PEPH; NEPH and CAT), and one pair represents positional isomerism (AMP and BMPEA). Pharmacologically, these sympathomimetics induce the release of catecholamines, such as dopamine, serotonin, and noradrenaline [3]. Figure 1 shows the chemical structures of the ARDs selected in this study.

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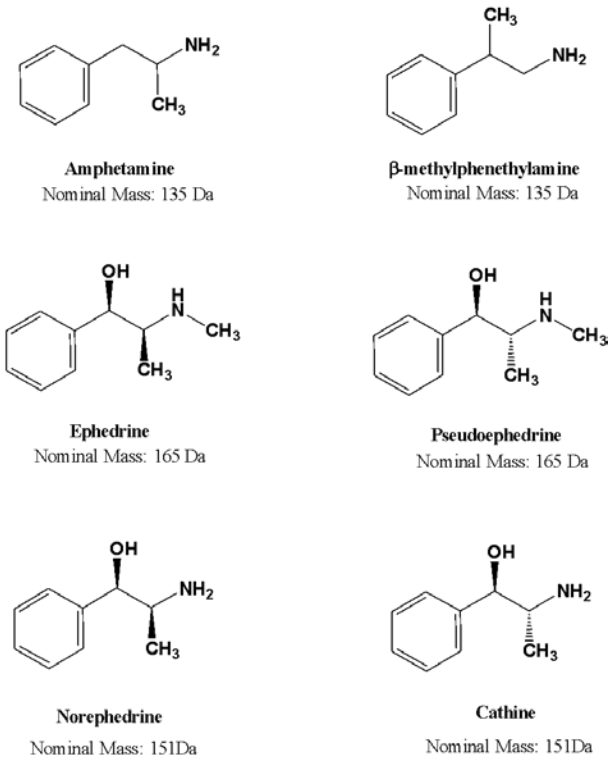

Figure 1. Chemical structures of amphetamine-related drugs included in this study.

ARDs are common compounds implicated in drug abuse in Saudi Arabia. This drug abuse plays an important role in early mortality due to traffic accidents, violence, and overdose [4]. Consequently, the entire Saudi society has been affected from abusing ARDs. Therefore, control of ARDs abuse is important. In forensic toxicology, analysis of ARDs may be utilized to identify those driving or performing other tasks under the influence of drugs, to clarify the manner and cause of death, and to identify individuals who have been exposed to drugs in the recent past. For purposes of estimation of the degree of drug toxicity, this analysis is best carried out by using blood samples, since blood drug concentrations are generally best correlated with the extent of toxicity.

The importance of isomeric form identification is growing, particularly in forensic and doping control situations. This is due to the fact that different isomers have varying levels of safety, efficacy, and legislation. The positional isomer of amphetamine, BMPEA, is currently uncontrolled, whereas amphetamine is a controlled substance. BMPEA can also be found in nutritional supplements for athletes and dieters [5-7]. Because of their positional isomers, their similar mass spectra can lead to incorrect identification [8]. Misinterpretation in this regard may result in a false conviction. In this study, the six selected ARDs included two pairs of diastereomers (NEPH and CAT, and EPH and PEPH) and one pair of positional isomers (AMP and BMPEA). Therefore, fragmentation patterns for each pair of isomers were expected to be very similar or indistinguishable.

Within the field of forensic toxicology, new analytical methods must undergo a process comprised of three stages prior to being adopted and incorporated within the laboratories' standard analytical methods. The three stages involve development, validation, and verification. It is crucial in forensic toxicological analysis to obtain reliable, consistent, and accurate measurements. Therefore, validation of the developed method is a prerequisite to analyzing actual samples in forensic casework. Validation involves performing a set of experiments to estimate the efficacy and reliability of an analytical method [9]. In forensic toxicological analysis, these experiments must be performed according to the most recent professional standards for the intended application. One example of such

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standards are those established by Scientific Working Group for Forensic Toxicology (SWGTOX) [9].

In this study, a pseudo-isocratic UPLC-qTOF-MS method for the analysis of amphetamine-related drug isomers in blood after extraction by mixed mode cation exchange (MMSPE) was developed and validated using the standards established by SWGTOX.

2. Materials and Methods

2.1. Experimental Materials

Standards for (±) amphetamine, (S,S)-(+)-pseudoephedrine, (1S,2R)-(+)-ephedrine-d3 HCl, and (±)-amphetamine-d11 were purchased from Cerilliant (Round Rock, TX, USA) as 1 mg/mL methanolic solutions and diluted as required. (R*,S*)-(±)-ephedrine HCl, DL-norephedrine, and (R)-(+)-β-methylphenethylamine were obtained from Sigma Aldrich (Oakville, Ontario, Canada) as 1 mg/mL methanolic solutions and diluted as required. (+)-Norpseudoephedrine hydrochloride (cathine hydrochloride) was purchased from LGC Standards (Manchester, NH, USA) as a 0.1 mg/mL methanolic solution and diluted as required. ACN, MeOH, and purified water, used in drug extraction and UPLC analysis, were of reagent grade and obtained from EMD Milipore (Billerica, MA, USA). Ammonium acetate was purchased from Mallinckrodt Baker Inc. (Phillipsburg, NJ, USA). Acetic acid and HCl were obtained from BDH (Radnor, PA, USA). Ammonium hydroxide, ammonium formate, and formic acid were purchased from Fisher Chemicals (Bridgewater, NJ, USA). Mixed-mode SPE (Oasis MCX, 30 mg) and FTPE (HLB Prime, 100 mg) were purchased from Waters (Milford, MA, USA). Blank human whole blood was obtained from Utak Laboratories Inc. (Valencia, CA, USA).

Methanolic combined working solutions of the analytes were made at different concentrations levels (20, 40, 200, 500, 800, and 1000 ng/mL) for the spiking of drug-free blood, and the preparation of calibration standards samples. Three internal standard (IS) solutions containing 20, 500, 1000 ng/mL of the deuterated analogues of ephedrine and amphetamine were also prepared in MeOH for determining matrix effects.

2.2. Sample Preparation and extraction

Spiked and drug-free blood or aqueous samples (250 µL) were mixed sequentially with 1 mL each of 0.1 M HCl and ACN. The mixtures were vortexed and centrifuged at 5000 rpm for 15 min at room temperature, and the supernatants were decanted into clean tubes. MMSPE was carried out by using Oasis MCX 96-well plates. The wells were conditioned with 1 mL of MeOH and equilibrated with 1 mL of water. The supernatants obtained from the pre-treatment step were loaded under gravity, and SPE wells were washed sequentially with 1 mL each of 0.1 M HCl, MeOH, and 5% NH4OH. The wells were dried under vacuum (-10 kPa) for 10 min, and the analytes were eluted with 1 mL of 5% NH4OH in MeOH (Figure 2). The eluates were evaporated to dryness under vacuum centrifugation at 30 °C, the residues were reconstituted in 200 µL of mobile phase A, and then underwent UPLC-qTOF-MS analysis (Figure 3).

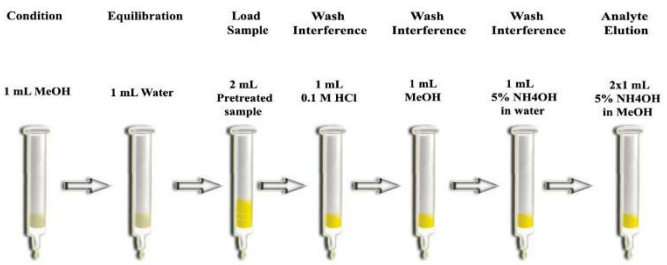

Figure 2. Schematic diagram of MMSPE extraction.

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Figure 3. Schematic diagram of the analytical method used in this study.

Drug-free blood or aqueous samples (250 µL) were diluted with 1 mL of MeOH. The mixtures were then mixed with 1 mL of ACN. The mixtures were vortexed and centrifuged at 5,000 rpm for 15 min at room temperature, and the supernatants were decanted into clean tubes. FPTE was carried out by using HLB Prime 96-well plates. The supernatants were directly loaded under gravity into the SPE wells. The eluates were collected and evaporated to dryness under vacuum centrifugation at 30°C. The dry residues were reconstituted in 200 µL of mobile phase A, and submitted for UPLC-qTOF-MS analysis (Figure 3).

2.3. Analytical methods

Chromatographic separations were obtained on an ACQUITY UPLC™ HSS T3 column (100 mm × 2.1 mm, 1.8 µm) maintained at 45°C. Mobile phase A consisted of water, 0.1% formic acid, and 5 mM sodium formate; mobile phase B was composed of ACN and 0.1 % formic acid. The mobile phase composition was controlled as follows: 0–1 min, 0% B; and 1–10 min, 5% B (pseudo-isocratic) for baseline resolution of the isomeric analytes; 10–11 min, 30% B, 11–12 min, 50% B; 12–13 min, 100% B; and 13–15 min 0% B. The flow rate was 0.5 mL/min, and the injection volume was 5 µL. Table 1 shows the optimized run method conditions

Mass spectrometry was performed on a Waters Acquity UPLC equipped with a Waters Xevo G2-XS-qTOF-MS (Waters, Medford, MA). Data was acquired in sensitivity mode using positive electrospray ionization with a resolution > 20,000 at full width half maximum. The acquisition range was *m/z* 50–601 using a scan time of 0.1 s. Capillary voltage and cone voltage were 0.8 kV and 20 V, respectively. The source temperature was 140°C, the desolvation gas flow rate was 900 L/h at 250°C, and the cone gas flow rate was 50 L/h. Data acquisition used the MSE mode, with low collision energy (4 eV) and high-energy ramp (10–40 eV). Mass correction was performed during acquisition using an external reference (lockspray) composed of 2 µg/mL leucine enkephalin (monitoring *m/z* = 278.1114) solution infused at a flow rate of 5 µL/min. Table 1 shows the Analytical parameters of the analytes.

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Table 1. Analytical parameters of the analytes (positive ionization mode).

Drug	Molecular Ion (m/z)	Fragmented Ion (m/z) (± 0.01)	Retention Time (min) (± 0.05)
Amphetamine−$\boldsymbol{d_{11}}$	147.1938	98.1078* /130.1653	8.76
Ephedrine−$\boldsymbol{d_3}$	169.1568	136.1195 /151.1433*	7.30
Norephedrine	152.1180	115.0736 / 117.0736 /134.0975*	5.21
Cathine	152.1180	115.0736 / 117.0736 /134.0975*	5.90
Ephedrine	166.1378	115.0556 / 117.0713 / 148.1140* / 149.1160	7.31
Pseudoephedrine	166.1378	115.0556 / 117.0713 / 148.1140* / 149.1160	8.00
Amphetamine	136.1219	91.0553 / 119.0868*	9.05
β-methylphenethylamine	136.1219	91.0553 / 119.0868*	9.58

*Quantifier ions

2.4. Method validation

Method validation was performed in accordance with the guidelines established by SWGTOX. Matrix Interferences (MIs) were evaluated to confirm the absence of substances that may interfere with analyte detection in blood matrices. Five different types of aged animal and human drug-free blood matrices were pretreated and extracted in triplicate by following the MMSPE pretreatment and extraction method. These extracted samples were analyzed for MIs by UPLC-qTOF-MS.

The term "Matrix Effects" (MEs) refers to the modification or interference of the instrument response, whether direct or indirect, carried on by the co-eluting substances present in the sample matrix [9]. MEs were evaluated using five different types of aged animals and human drug-free blood matrices. All blood samples were pretreated and extracted in triplicate by following the MMSPE pretreatment and extraction method. The extracted drug-free samples were spiked with combined working standard and IS solutions in triplicate at concentrations of 20 (low), 500 (medium), 100 (high) ng/mL. Spiked samples and corresponding neat standards were analyzed, and their instrumental responses were used to determine the magnitude of ME by using the following equation:

$$\%\boldsymbol{ME} = \frac{Response_{spiked\ post-extracted\ sample}}{Response_{standard\ (working)solution}}\ x\ 100$$

Where, ME less than 100 indicates suppression, and ME greater than 100 indicates enhancement. The acceptable range of ME is considered as 100 ± 25 = 75–125. MEs at each concentration level are represented as percentage increase or decrease in the peak areas of analytes in the samples relative to those of analytes in the neat standards.

Recovery refers to the fraction of original analyte mass that is carried through the extraction process and is present in the final extract. It is determined as the ratio of analyte response in an extract to that of a drug-free extract when the sample is spiked with the same mass of the analyte. Two different types of aged animal drug-free blood matrices were evaluated for recovery in triplicate at three concentration levels (low, medium, and high) in pre- and post-extraction spiked samples. Both samples were processed by following the MMSPE pretreatment and extraction method. The samples were spiked with combined working standard and IS solutions in triplicate at concentrations of 20 (low), 500 (medium), and 1000 (high) ng/mL either before (pre-extraction spiked) or after (post-ex-

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traction spiked) extraction. The spiked samples were analyzed, and their instrumental responses were used to determine the magnitude of recovery by using the following equation:

$$\%\boldsymbol{RE} = \left(\frac{Response_{pre-extraction\ spiked\ sample}}{Response_{post-extraction\ spiked\ sample}}\right) \times 100$$

Carryover was evaluated by analyzing extracts of 250 µL of drug-free blood (n = 3) after analyzing a high-concentration calibrator (1000 ng/mL, n = 3) of the analytes. Both the samples were pretreated and extracted by the MMSPE extraction method.

Calibrators (250 µL) were prepared in drug-free aged bovine blood matrix at concentrations of 20, 40, 200, 500, 800, 1000 ng/mL by using combined working standard solutions each containing 125 ng of ISs. All samples were pretreated and extracted by the MMSPE extraction method and analyzed by UPLC-qTOF-MS. Quantification was performed by measurement of the ratio of peak areas of the analytes relative to those of the corresponding deuterated analogs in the specific EICs; deuterated AMP was used for quantifying AMP and BMPEA, and deuterated EPH was used for quantifying EPH, PEPH, NEPH, and CAT. Calibration curves were constructed and assessed using quadratic regression (considered acceptable if $R2 \geq 0.99$) of peak area ratios versus concentration on each of five different days. Each calibration curve was constructed using six calibrators in triplicate for each analyte. Furthermore, a batch of blind samples were analyzed in triplicate at two concentration levels along with the calibrators for purposes of assessment of analytical bias.

The working concentration range of the method was 20–1000 ng/mL for all analytes. The LOD was administratively defined as 20 ng/mL by using the lowest non-zero calibrator method (the calibrator with lowest concentration assayed with response that met precision criteria), due to the lack of toxicological significance of the analyte compounds at blood concentrations below 20 ng/mL. Fifteen samples with analytes at a concentration of 20 ng/mL were used to identify the LOD. All these samples were pretreated and extracted by following the MMSPE method. Similarly, the LOQ was also identified by following the same method for identifying LOD.

Analytical precision was measured as the coefficient of variation (%CV) of triplicate measurements at the assayed concentration range on each of five different days. It was considered acceptable when the %CV was ≤ 20%. Bias was determined after blinded analysis of triplicate samples at two different concentrations of each analyte per run. Bias was considered acceptable when the measured concentration was within 20% of the theoretical concentration.

Analyte stability within the autosampler (maintained at 10°C) of the UPLC-qTOF-MS was evaluated by repeated injection of extracted samples at three different concentration levels (40, 500, and 1000 ng/mL; n = 3) after 0, 12, 24, and 36 h. Analytes were considered stable if the deviation in analyte response was within 20% of the response of the corresponding sample at t= 0 h

2.4. Data Processing

Analyte stability within the autosampler (maintained at 10°C) of the UPLC-qTOF-MS was evaluated by repeated injection of extracted samples at three different concentration levels (40, 500, and 1000 ng/mL; n = 3) after 0, 12, 24, and 36 h. Analytes were considered stable if the deviation in analyte response was within 20% of the response of the corresponding sample at t= 0 h

3. Results

3.1. Matrix Interferences and effects

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The EICs at the retention times of the analytes in aged blood sheep, bovine, and three human blood matrices exhibited no Matrix Interferences.

Matrix effects (suppression/enhancement) were less than 25% for all analytes tested in the five different blood matrices. Table S1 (A-E) show the Matrix effects values of all analytes in aged bovine, sheep, and human blood matrices, respectively;

3.2. Recovery and carryover

Recovery ranged from 60–90% for all analytes in aged bovine and sheep blood matrices (Figure S1 and Table S2). Carryover was evaluated by analysis of three drug-free aged animal blood extracts directly after analyzing the high concentration calibrator (1,000 ng/mL, n = 3) samples. No carryover was observed upon visual inspection of the chromatograms and after the analysis of EICs.

3.3. Analytical calibration

Analytical response ratios were fit to calibrator concentrations using quadratic regression equations over a range of 20–1,000 ng/mL. Strong correlations (R2 > 0.99) were observed on all five days. Table S3 shows the averaged calibration curve regression equations and correlation coefficients for all analytes. Averaged quadratic calibration curves are shown in Figure 4A (NEPH and CAT), 4B (EPH and PEPH), and 4C (AMP and BMPEA). The LOD and LOQ were determined to be 20 ng/mL for all analytes. Intra- and inter-day precision were 1.00–18.30% and 6.60–19.70%, respectively; they were deemed acceptable. The accuracy of the method was also acceptable (-11–18.25%). Table S4 summarizes the parameters determining analytical performance.

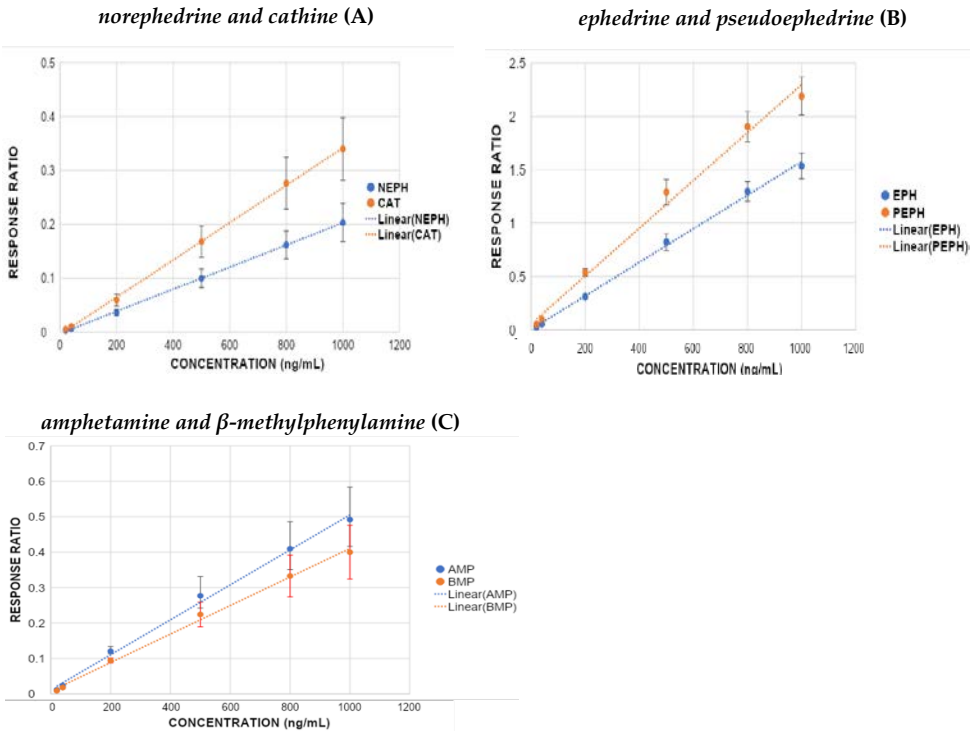

Figure 4. Averaged quadratic calibration curves of (A) norephedrine and cathine, (B) ephedrine and pseudoephedrine, and (C) amphetamine and β-methylphenylamine.

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3.4. Stability of Analytes in Autosampler

The stability of the analytes in the autosampler was assessed at three different concentrations over 36 h. For all analytes, there was no change in response ratio in excess of 20% of the initial response (t = 0 h), indicating that they remained stable while on the instrument waiting to be injected (Table S5).

4. Discussion

Drug analyses in forensic toxicology are performed to identify the use of drugs of forensic relevance or whether the subject was under the influence of certain drugs during a particular period [10]. Results of these analyses must be accurate and reliable to avoid any false positive or false negative results that could lead to severe consequences. Therefore, the utilization of highly sensitive and selective hyphenated analytical techniques, such as liquid chromatography-mass spectrometry (LC-MS), is necessary to qualitatively and quantitatively analyze drugs in blood samples [11].

This study was conducted to develop and validate a qualitative and quantitative method to determine ARDs in blood by UPLC-qTOF-MS after extracting the samples by MMSPE. Validation of the analytical method was based on standards established by SWGTOX [9]. To optimize the extraction process, several experiments were performed, including the evaluation of different precipitation agents, modes of SPE, and conditions of UPLC-qTOF-MS analysis. Samples of blood were precipitated using MeOH and ACN, alone or in combination. Based on visual inspection of the supernatant (i.e., clarity), ACN was deemed to be the most suitable precipitation agent in the pretreatment step of blood samples. After evaluating two types of SPE platforms for the extraction step, MMSPE was found to be superior to MIP-SPE because it did not elicit any interference to response associated with key analytes.

Prior to the development of hyphenated LC-MS, the GC-MS method was the ideal standard analytical method for detecting ARDs in blood. Kudo et al. described a method for qualifying and quantifying 13 ARDs in blood using GC-MS with an enhanced polymer column. This method was developed after the analytes were extracted from blood using SPE and derivatized via acetalization. This study did not focus on isomeric analytes [12]. The PEPH isomer was successfully separated from the EPH diastereoisomer in our method.

The separation of structurally isomeric analytes requires an extended runtime to ensure full baseline resolution of the analytes, especially when they yield the same fragment ions. In such an analytical assay, mobile phase composition is maintained isocratic (constant mobile phase composition) or "pseudo-isocratic", where the mobile-phase composition gradient is extremely shallow [13, 14]. Chołbiński *et al*. reported an isocratic method to resolve amphetamine (AMP) from its structural isomer, beta-methylphenethylamine (BMPEA), using UPLC-MS/MS with a runtime of 9.5 min [15].

In our study, the six selected ARDs included two pairs of diastereomers (NEPH and CAT, and EPH and PEPH) and one pair of positional isomers (AMP and BMPEA). Therefore, fragmentation patterns for each pair of isomers were expected to be very similar or indistinguishable. To overcome this analytical challenge, chromatographic resolution of the ARDs was crucial. Accordingly, a pseudo-isocratic elution method was developed in which the composition of the UPLC mobile phase was varied at very shallow gradients (from 0% B to 5% B over 9 min) to facilitate the complete baseline resolution of the isomeric analytes.

Rapid, alternating acquisition of MS spectra at low (LE) and high (HE) collision energies during qTOF-MS analysis was performed in the MSE mode. Ions from intact molecules were generally and predominantly detected at LE, whereas more extensive fragmentation data was acquired at HE. In a single injection, this technique enables the acquisition of ions from precursor molecules and their fragment ions (Hernández et al., 2011). Low capillary voltage (0.8 kV) facilitated the detection of low-molecular-mass ARDs (135–168

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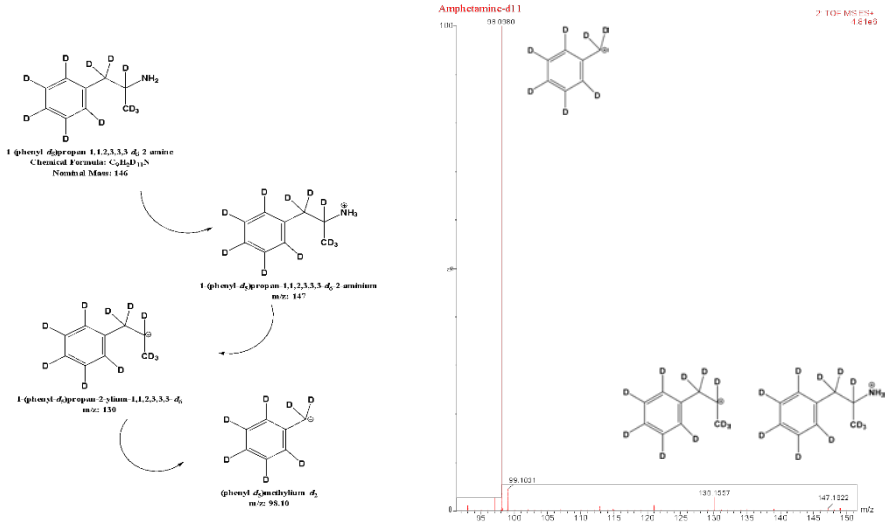
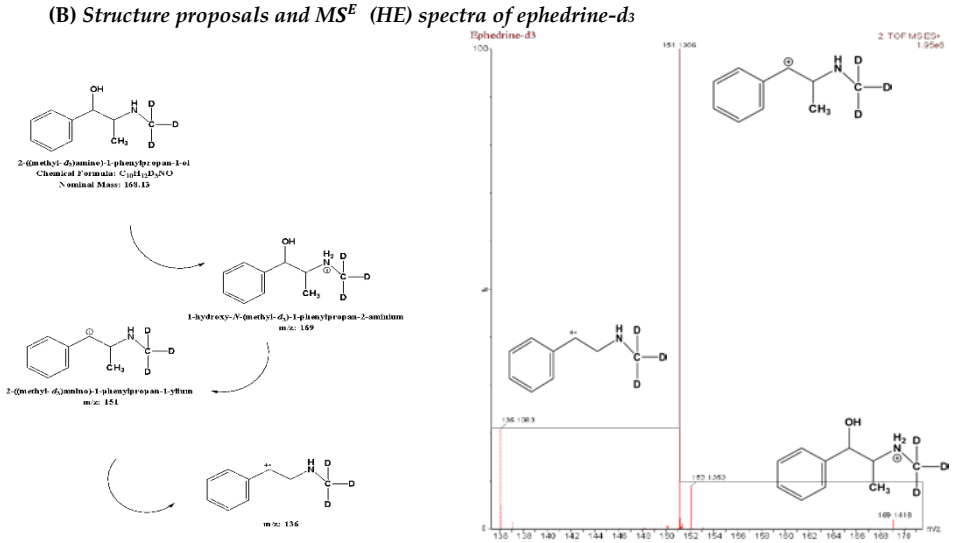
Da) with optimum sensitivity. Similarly, the cone voltage was also set to a low voltage (20 V) as shown in Table 2. The analytical method was optimized for these parameters (Table 1).

Both isomer pairs, NEPH and CAT, and EPH and PEPH, formed [M+H]+ ions with *m*/*z* 152 and 166, respectively. Consequently, by losing water from their molecular ions [M+H-H2O]+, the isomer pairs formed fragments with *m*/*z* 134 and 148, respectively. NEPH and CAT also yielded [M+H-H2O-NH2]+ with *m*/*z* 117 after subsequently losing ammonia, whereas EPH and PEPH yielded [M+H-H2O-NH-CH3]+ with *m*/*z* 117 after losing methylamine. Moreover, [M+H-H2O-NH2-H2]+ with *m*/*z* 115 was formed by both isomer pairs due to the loss of H2 [16]. Ephedrine-d3 yielded [M+H]+, [M+H-H2O]+, and [M+H-H2O-CH3]+ with *m*/*z* 169, 151, and 136, respectively [16]. AMP and BMPEA displayed the same fragmentation pattern. They formed [M+H]+ with *m*/*z* 136. Subsequently, they formed [M+H-NH2]+ with *m*/*z* 119 by losing ammonia and tropylium ion [M+H-NH2-CH3-CH]+ with *m*/*z* 91 as a result of a β-C-C cleavage. Amphetamine-d11 formed [M+H]+, [M+H-NH2]+, and [M+H-NH2-CD3-CD]+ with *m*/*z* 147, 130, and 98, respectively. Figure 5 (A-E) shows chemical structure proposals for molecular ion fragmentation acquired at high collision energy (HE) during qTOF-MS analysis for amphetamine-d 11, ephedrine-d 3, phenylpropanolamine (norephedrine and cathine), and methyl phenylpropanolamine (ephedrine and pseudoephedrine).

(A) ***Structure proposals and MS^E (HE) spectra of amphetamine-d*** $_{11}$



(B) ***Structure proposals and MS^E (HE) spectra of ephedrine-d*** $_3$



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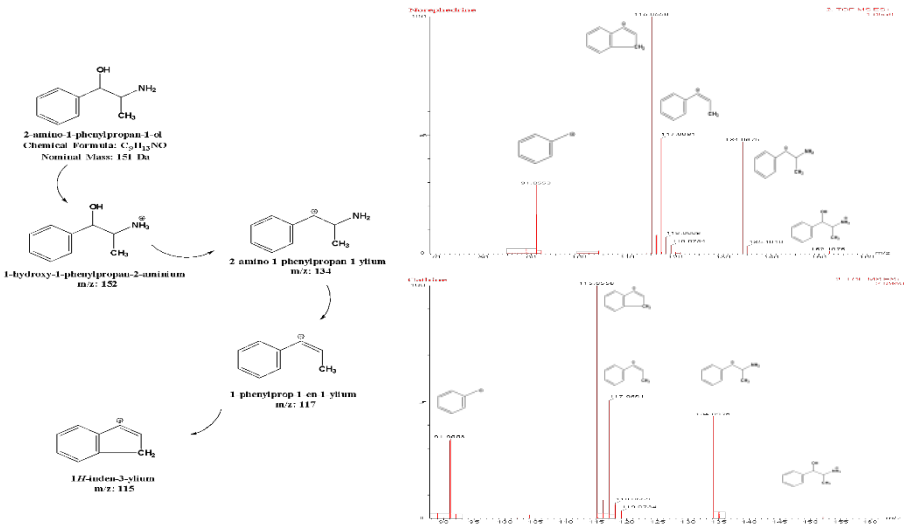
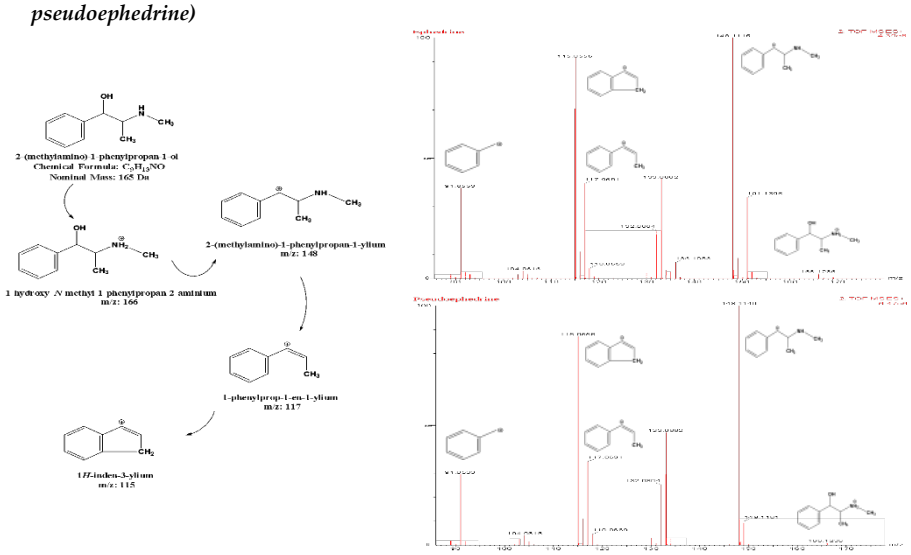
(C) ***Structure proposals and MS^E (HE) spectra of phenylpropanolamine (norephedrine and cathine)***



(D) ***Structure proposals and MS^E (HE) spectra of methyl phenylpropanolamine (ephedrine and pseudoephedrine)***



(E) ***Structure proposals and MS^E (HE) spectra of amphetamine***

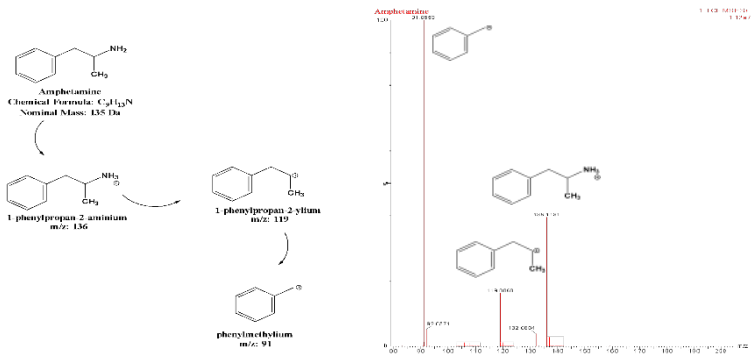


Figure 5. Chemical structure proposals for molecular ion fragmentation acquired at high collision energy (HE) during qTOF-MS analysis for amphetamine-d 11 (A), ephedrine-d 3 (B), phenylpropanolamine (norephedrine and cathine) (C), methyl phenylpropanolamine (ephedrine and pseudoephedrine) (D), and amphetamine (E).

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The developed method was validated by evaluating matrix interferences, matrix effects, recovery, carryover, autosampler stability, and calibration to measure bias, intra- and inter-day accuracy, and precision. Five types of drug-free blood matrices were extracted and analyzed without the addition of ISs. Each sample was analyzed to confirm the absence of matrix interferences by monitoring the quantifier and qualifier ions of the analytes of interest at their respective retention times; no matrix interferences was detected in the five blood matrices.

Table 2. Optimized UPLC-qTOF-MS method parameters.

Chromatography	
Liquid chromatography system:	Waters ACQUITY UPLC
Column:	Waters ACQUITY® HSS T3 (2.1 x 100 mm, 1.8 µm)
Column temperature:	45 °C
Injection volume:	5 µL
Solvent A:	5 mM ammonium formate, adjusted to pH 2.9 using formic acid
Solvent B:	Acetonitrile containing 0.1% (v/v) formic acid
Gradient:	0 % solvent B (0-1 min)
	5 % solvent B (1-10 min)
	5-30 % solvent B (10-11 min)
	30-50 % solvent B (11-12min)
	50-100 % solvent B (12-13min)
	100-0 % solvent B (13-14 min)
	0 % solvent B (14-15 min)
Flow rate:	0.5 mL/min
Mass Spectrometry	
Mass spectrometer:	Waters Xevo G2-XS QTof
Ionizations mode:	Electrospray +ve
Capillary voltage:	800 V
Cone voltage:	20 V
Cone gas:	50 L/h
Desolvation temperature:	250°C
Desolvation gas:	900 L/h
Source temperature:	140°C
Data acquisition:	MSE centroid (data independent acquisition)
Function 1:	4 eV
Function 2:	Ramp 10-40 eV
Mass ange:	50 to 601 Da
Resolution:	> 20,000 @ 278 *m/z* (resolution mode)
Lock Spray:	leucine enkephalin = 278.1114 *m/z*
Lock Spray Infused Rate:	5 µl/min

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Changes (enhancement or suppression) of analyte responses due to matrix effects must be less than 25%as per SWGTOX guidelines. matrix effects of the five different blood matrices on the responses of all analytes at all concentration levels assayed were less than 25% (with %CV ≤ 20%). The majority of the matrix effects values were negative for bovine, and human source 1, 2, and 3 blood matrices, indicating ion suppression. However, ion enhancement was observed in the sheep blood matrix as shown by positive matrix effects values. Recovery was evaluated using aged bovine and sheep blood matrices; it was 65–90% for all analytes extracted from bovine blood, whereas it was 60–90% for all analytes extracted from sheep blood at all concentration levels (with %CV ≤ 20%). Low recovery values, especially at low analyte concentrations, were observed. These low recovery values might be due to the loss of analytes because of the polarity and volatility of ARDs. The deactivation of any utilized glass apparatus during the extraction and preventing the protonated amine group of ARDs from interacting with the hydroxyl group of glass silicone could increase analyte recovery and reduce matrix effects. This remedial step was not incorporated in this study due the high cost of silanized glass tubes. Therefore, further experiments are required to verify its benefits.

Analyte carryover may compromise the accuracy of qualitative or quantitative analysis. ARDs were evaluated for carryover by analyzing drug-free blood extracts immediately following the analysis of the corresponding upper calibrator (1000 ng/mL) in triplicate. None of the analytes in this study displayed carryover effects.

Accuracy and precision were determined by constructing calibration curves for each analyte on each of five separate days and running two blind samples (one high (640-960 ng/mL) and one low (32-48 ng/mL unknown concentration samples) with each curve. The curves were produced using calibrators prepared in triplicate at 20, 40, 200, 500, 800, and 1000 ng/mL. The LOQ was determined to be the lowest point on the curve that demonstrated a precision of ≤ 20% and an S/N ratio ≥ 10. The LOD was determined to be equal to the LOQ as the lowest concentration that was measured on the curve (the lowest non-zero calibrator method). R2 values (0.999–1) that were observed for each curve showed a good fit; each curve was fit with a quadratic regression equation.

The concentration of each unknown blind sample was calculated using the equation of the line of best fit, and bias was determined by comparing the calculated and theoretical concentrations by using the following equation:

$$Bias\ (\%)\ at\ Concentration_x = \left[\frac{Grand\ Mean\ of\ Calculated\ Concentration_x - Theoretical\ Concentration_x}{Theoretical\ Concentration_x}\right] \times 100$$

Bias values up to 20% are permissible as per SWGTOX guidelines [9]. The high and low concentration blind samples for all analytes exhibited acceptable bias values, indicating that the method is reliable at both the high and low ends of the curve. Bias results of 10 blind and calibrator samples analyzed on five separate days are shown for each analyte in Table S4.

Precision, expressed as %CV, is the closeness of agreement between a series of measurements obtained from multiple samples of the same homogenous sample population [9]. Imprecision can lead to inaccurate quantitative results. SWGTOX guidelines state that the %CV shall not exceed 20% at any concentration level. Intra- and inter-run precisions were calculated. Intra-run precision was calculated using the values obtained in each run after triplicate analyses at each concentration as follows:

$$Intra-run\ \%CV = \left[\frac{Standard\ deviation\ of\ a\ single\ run\ of\ samples}{Mean\ calculated\ value\ of\ a\ single\ run\ of\ samples}\right] \times 100$$

Inter-run precision was calculated using the values obtained in each run after triplicate analyses at each concentration as follows:

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$$Inter-run\ \%CV = \left[\frac{Standard\ deviation\ of\ the\ grand\ mean\ for\ each\ concentration}{Grand\ mean\ for\ each\ concentration}\right] \times 100$$

The %CV for all analytes at all six concentrations were within the acceptable precision limit (≤ 20%). Table S4 presents the results of the accuracy and precision of the assay.

The stability of each analyte while samples resided in the autosampler was assessed. Stability is defined as the ability of an analyte to resist chemical change in a matrix under specific conditions for given time intervals [9]. It is a measure of the time for which an analyte can remain under those conditions before the interpretation of its concentration is affected; a change in response beyond ± 20% from the initial response indicates a loss of analyte stability. The relative change in response was measured at three concentrations (40, 500, and 1000 ng/mL) in triplicate at 0, 12, 24, and 36 h. The samples were maintained in the autosampler at 10°C. All analytes were stable throughout the period of evaluation as shown in Table 9, indicating that ARDs can reliably be quantified for at least 36 h under the autosampler conditions used here. The research presented here used aged blood in developing a method for the identification and quantification of ARDs. This study was performed on aged animal blood matrices because of our limited access to aged human blood. This type of study should be expanded to include aged human blood.

5. Conclusions

This work involved development and validation of a method for quantitative analysis of ARD isomers by UPLC-qTOF-MS in blood after extraction by MMSPE. The method was validated according to the standard practices of SWGTOX. The validation experiments demonstrated that the assay has acceptable accuracy and precision for use in forensic toxicology. In addition, the study proves that UPLC-qTOF-MS is an effective method for conducting both qualitative and quantitative analyses of ARD isomers in blood samples. Because of this, it has the potential to be applied in screening and quantification studies in the fields of doping control and forensic toxicology.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1: Recovery (%) of amphetamine-related drugs, including two deuterated analogues at three different concentrations from extract of spiked (A) aged bovine whole blood and (B) aged sheep whole blood.; Table S1: Evaluation of matrix effects (%) of aged bovine whole blood (A), aged sheep whole blood (B), human whole blood Source 1(C), human whole blood Source 2(D), and human whole blood Source 3(E) on amphetamine-related drugs and deuterated analogues; Table S2: Evaluation of recovery (%) of amphetamine-related drugs and deuterated analogues from aged bovine blood (A) and aged sheep blood (B).; Table S3: Averaged curve regression equations and correlation coefficients of the analytes in aged bovine blood; Table S4: Summary of analytical performance parameters; Table S5: Analyte stability data for amphetamine-related drugs at three different concentrations while.

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