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Original Article

LC-MS Method Development and Optimization for Small Drug Analysis in Urine

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ABSTRACT

Both amphetamine and methamphetamine are considered to be illegal chemicals, and hence, the purchase, possession, and use of these drugs is forbidden in many nations. Within the fields of forensic and clinical toxicology, there has been a recent uptick in the detection and quantification of illicit substances within urine samples. **Objective:** To detect and quantify both drugs in urine samples utilizing caffeine as an internal standard with an optimized liquid-liquid extraction procedure. **Methods:** An alternative rapid and efficient method of liquid chromatography – electron spray ionization – Tandem mass spectrometry (LC – ESI – TMS) was developed and optimized. The chromatographic separation was carried out using an isocratic high-performance liquid chromatography (HPLC) system, and the eluent that was applied was a mixture of 20% acetonitrile and 80% buffer with a pH of 2.6 that included 10mM ammonium acetate and 0.1% trifluoroacetic acid. The run duration was 9 minutes, and the detection was accomplished at 210 nm with a flow rate of 1 mL/min utilizing triple quadruple MSMS to validate ionic transitions following direct infusion and fragmentation of analytes. **Results:** An excellent linearity was seen in the calibration curves of amphetamine and methamphetamine in urine samples across the concentration range of 0-10 mg/L, with a regression coefficient of 0.91 and 0.97, respectively, for each of these substances. **Conclusions:** More compounds are able to be identified in urine as chromatographic techniques, such as high-performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS), continue to improve in terms of their sensitivity.

INTRODUCTION

The abuse of drugs and the development of a reliance on them have a wide range of negative effects on society, including increased rates of criminal activity, social unrest, and mortality [1]. Amphetamine is a chemical substance that is also known as alpha-methylphenethylamine [2]. It is a member of the phenylethylamine family of stimulants, which are known to have a significant impact on the central nervous system. This medicine is often prescribed to patients suffering from attention deficit hyperactivity disorder (ADHD), as well as obesity and sleeplessness [3]. A greater number of young people are becoming intoxicated with amphetamines and methamphetamines, which draws

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the attention of the media all over the globe [4]. This is due to the fact that a significant number of fatalities and hospitalizations are caused by the misuse of Ecstasy at parties and clubs. In this context, the Department of Public Health has to create methods for prevention and control [5]. The examination of addictive substances in biological matrices presents a number of difficulties, one of which is the selection of an extraction technique that produces samples that are pure and highly concentrated [6]. After the sample treatment has been decided upon, the sort of analytical equipment that will be utilized is the next step that has to be taken [7]. Due to their sensitivity, high accuracy, and the use of modest quantities of solvents and samples, a combination of GC-MS with analyses utilizing biological matrices is thus an effective detection approach [8]. However, in order to improve the GC-MS's chromatographic capabilities, the sample must often be derivatized before the analysis is performed [9]. Although there have been many advancements in extraction methods over the years, the liquid-liquid extraction (LLE) approach stands out as a pioneering technology. Using liquid chromatography and electrospray ionization tandem mass spectrometry, [10] established a technique for the identification of amphetamine and methamphetamine from blood and urine samples. This approach was published in the journal Analytical Chemistry. In recent years, amphetamine and methamphetamine, along with a broad variety of other small compounds found in biological matrices, have been efficiently examined utilizing liquid chromatography (LC) connected to mass spectrometry (MS). The fact that LC-MS/MS does not call for any sample derivatization contributes to the fact that it has garnered a significant amount of interest [11]. Because of this, one of the most important methods is called liquid chromatography tandem mass spectrometry, or LC-MS/MS for short. It is used to analyze pharmaceuticals that have been found in bodily fluids [12]. The preparation of the samples has to be improved so that the analysis of amphetamines and methamphetamines may be more accurate and completed in a shorter amount of time. The traditional LLE method was used throughout this investigation [13, 14]. Extraction is the technique of separation that may be used to separate one or more components from a mixture and to concentrate the sample. In general, extraction is the method that is employed [2, 15]. When using LLE, the process of separation includes the movement of a solute from one solvent to another. This movement may take place in either two immiscible or two partly miscible solvents [16]. By integrating liquid chromatography with liquid chromatography–electrospray ionization tandem mass spectrometry (LC–ESI–MS/MS), the researchers in this study were able to establish a technique that was both easy and sensitive for determining the presence of amphetamine and methamphetamine in urine samples [17, 18]. For the purpose of drug detection, triple quadruple MSMS was used to validate ionic transitions after direct infusion and fragmentation of analytes [19]. Additionally, the conditions for LLE and LC-MS/MS detection were researched in order to achieve the highest possible level of performance [3].

METHODS

In order to develop and optimize an HPLC technique, an internal standard of caffeine at a concentration of 20 mg/L as well as unextracted samples of amphetamine and methamphetamine at concentrations of 100 mg/L each were employed. When doing a liquid-liquid extraction, dichloromethane was employed as the extracting solvent. Deionized water, 2M sodium hydroxide solution, and 2M hydrochloric acid were used when attempting to modify the pH of the sample. The quaternary pump was used for the HPLC analysis, and the instrument was an Agilent 1260 infinity II. The eluent for the HPLC that was used consisted of 20% acetonitrile combined with 80% buffer that had a pH of 2.6 and included 10mM ammonium acetate and 0.1% trifluoroacetic acid. The cycle duration was kept at 9 minutes, the eluent flow rate was kept at 1.0 milliliters per minute, and the detector wavelength was kept at 210 nanometers. In the system, there was a C18 reverse-phase partition column from Agilent Technologies called the Infinity Poroshell 120EC-C18. This column had dimensions of 150 millimeters in length and 4.6 millimeters in internal diameter, and it was filled with octadecyl (C18) coated porous silica beads. The GCMS apparatus consisted of a 7890A gas chromatograph (GC) system and a 5975C VL mass spectrometer (MSD) with triple axis detector (mass spec detector). Mixed standards of amphetamine, methamphetamine, and the internal standard caffeine were created in various concentrations, including 2 mg/L, 4 mg/L, 6 mg/L, 8 mg/L, and 10 mg/L respectively. Following the preparation of three duplicate aliquots of each standard, an appropriate pH-based liquid-liquid extraction was performed, and then the sample was analyzed using HPLC. After that, the solutions were injected for analysis, and the chromatograms corresponding to those injections were recorded. Following the construction of calibration curves based on the results acquired from HPLC, the quantification of drugs in the urine sample extracts as well as the determination of their concentration in the original urine samples were carried out. For the purpose of verifying the identification of the samples, a comparison of the chromatograms of the blank urine samples with those of the standard drug samples (amphetamine and

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methamphetamine) was carried out. The LLE procedure was carried out for the GCMS analysis without first evaporating the sample to dryness and then reconstituting it with mobile phase._A sample of 1 milliliter of pee was collected in 15 milliliter falcon tubes, and the pH was altered to acidic, basic, and neutral states by the addition of 2 milliliters of HCl, 2 milliliters of NaOH, and deionized water, respectively. After adjusting the pH of the urine sample to 1 milliliter, 500 microliters of dichloromethane were added, and the mixture was given a minute to be vortexed. After that, the aqueous layer was extracted with dichloromethane a second time. Finally, the bottom layer was taken out. The two extracts were mixed together and then dried out by evaporating them under an atmosphere of nitrogen. After that, 1 milliliter of a mixed mobile phase was used to re-create the sample.

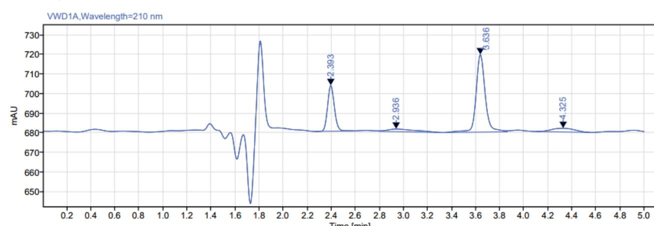
RESULTS

A series of repeated injections and subsequent analyses were carried out under a wide range of settings in order to determine the HPLC operating parameters that gave the best results. Following completion of the optimization process, the amounts of time required for the retention of amphetamine, methamphetamine, and caffeine were determined to be 3.6 minutes, 4.3 minutes, and 2.3 minutes, respectively. The following HPLC settings were optimized in order to facilitate the identification of amphetamine in sample A and methamphetamine and amphetamine in sample B, respectively: 80:20 (v/v), buffer (10mM Ammonium Acetate, 0.1% Trifluoroacetic acid, pH - 2.6) – Acetonitrile, wavelength 210 nm, which produced the highest absorption by all analytes, and the ideal flow rate of 1 mL/min.

At addition, amphetamine and methamphetamine were found at varying amounts in the urine samples that were extracted. Extraction efficiency was calculated at pH 12 by using the theoretical equation given below.

$$EE = \frac{\text{(absorbance of drug extracted from urine sample)}}{\text{(absorbance of drug standard)}} \times 100$$

Hence, the extraction efficiency of amphetamine in urine samples was 61% and those of methamphetamine was 51%. The recorded chromatograms for suspect urine sample A and sample B were given in Figure 1. The data depicts clear vision of presence of amphetamine in samples A (R_T 3.6 min) and sample B (R_T 3.6 min) while methamphetamine in sample B (R_T 4.2 min).



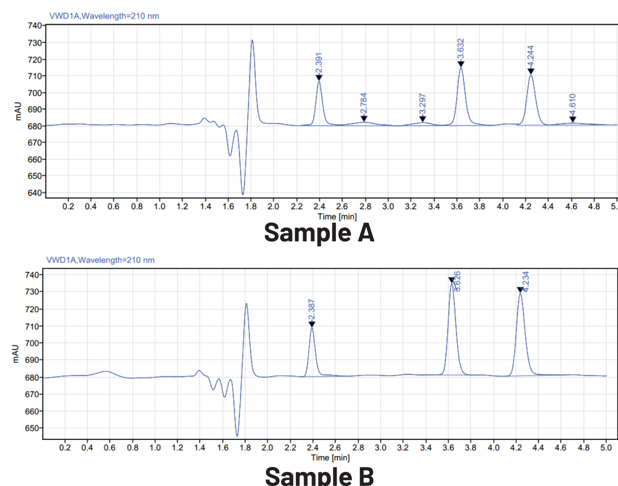


Figure 1: Chromatograms of urine sample A and urine sample B detecting the presence of amphetamine and methamphetamine

For concentration range from 0-10mg/L, equation for line of best fit and value of R^2 for:

(I) Amphetamine

$y = 0.2733x + 0.1735$; $R^2 = 0.9088$

(II) Methamphetamine

$y = 0.283x + 0.23$; $R^2 = 0.9683$

By interpolating the calibration curve, concentration of amphetamine from urine sample A was 7.6 mg/L and concentration of methamphetamine and amphetamine from urine sample B was 4.3 mg/L and 5.2mg/L respectively.

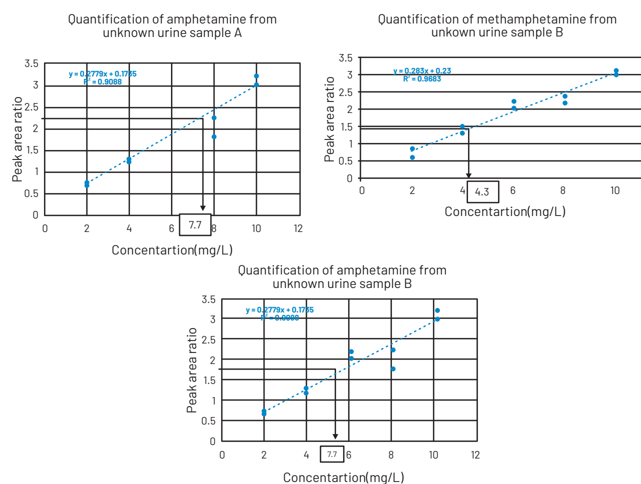


Figure 2: Calibration graphs of amphetamine and methamphetamine

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The presence of amphetamine and methamphetamine was detected in the total ion chromatograms of both sample A and sample B. The retention durations of 4.4 minutes for amphetamine and 4.6 minutes for methamphetamine indicated their existence. The m/z value 44 was acquired for both sample A and sample B from the mass spectrum, which validates the presence of amphetamine in both samples. The m/z value 58 was found for sample B, which indicates the presence of methamphetamine in that sample. Tentatively Identified Compound (TIC) of sample A and sample B was given in Figure 3.

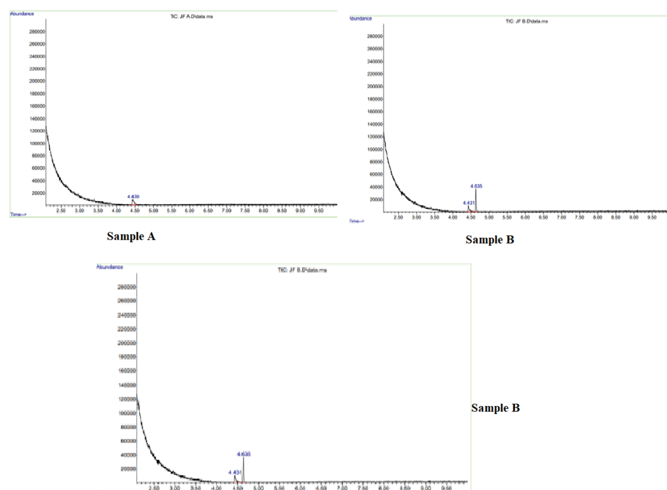
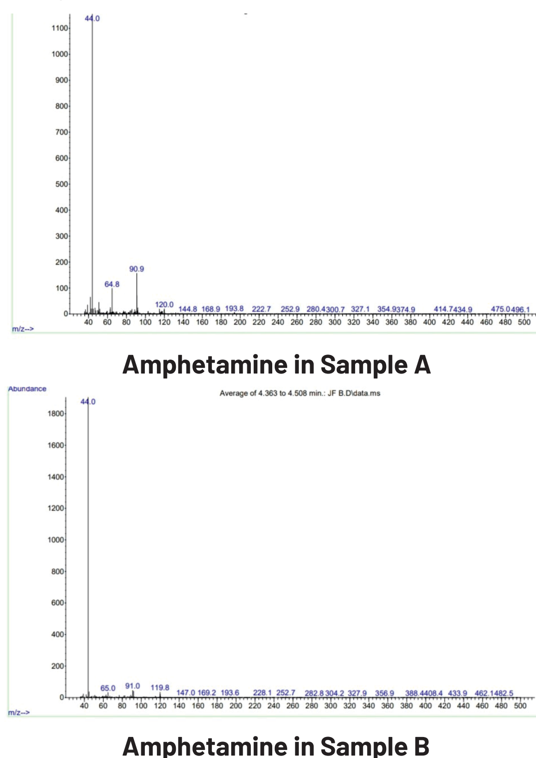


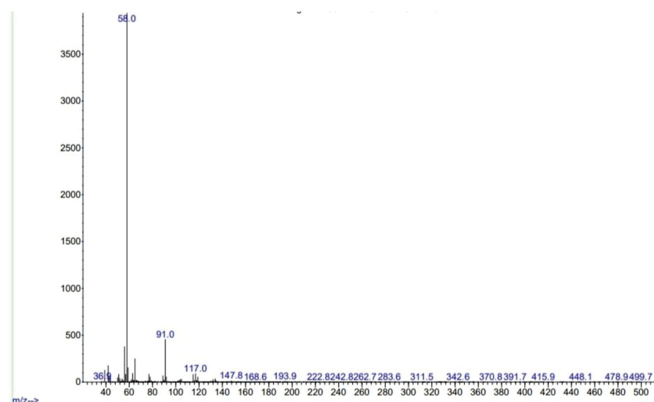
Figure 3: TIC of sample A and B

The mass spectrum of sample A and those of sample B was given in Figure 4.



Amphetamine in Sample A

Amphetamine in Sample B



Amphetamine in Sample C

Figure 4: MS peak of sample A and sample B

DISCUSSION

The purpose of the current work is to create a technique of LC-MS for the analysis and identification of amphetamine and methamphetamine using caffeine as an internal standard, with the end goal of determining the concentration of drugs in urine samples whose contents are unknown. LLE was preferred because it was reliable and did not require any particular instrumentation for sample preparation [13]. The drugs that were used in this experiment were basic in nature; as a result, liquid-liquid extraction with dichloromethane as the extracting solvent was carried out for the urine samples with a pH of 10 [10]. The UV-Vis spectrophotometer was used to determine that a wavelength of 210 nm should be used for the detector. Altering the flow rate and mobile phase composition in the experiment, such as 75:25 (v/v), 80:20 (v/v), etc., led to the conclusion that the conditions for the experiment had been optimized. The values for all of the other parameters, such as the run duration (9 minutes) and the detector wavelength (210 nm), were held steady. When the mobile phase composition was kept at 80:20 (v/v), buffer (10mM), it was possible to get peak separation that was satisfactory. On the basis of data from past publications, it was anticipated that clinical samples would have urinary concentrations of up to 4,000 mg/L [20-23]. By interpolating the calibration curve, we were able to determine the concentrations of amphetamine and methamphetamine in two unknown urine samples, A and B. The amphetamine concentration in A was 7.7 mg/L, while the methamphetamine concentration in B was 5.2 mg/L. Both of these concentrations were acquired from the samples of pee. The same drugs were previous recorded in various studies like Bergan *et al.*, and Muller-Serieys *et al.*, where the maximum urinary concentrations ranged from 1050.3 mg/L to 4378.9 mg/L [21, 24-26]. The total ion chromatograms, or TICs, of samples A and B both indicate the presence of amphetamine in both samples, but the TIC of sample B also

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reveals the presence of methamphetamine in that sample. The discovery was verified using mass spectrometry (MS) [12]. The current approach may be used for the analysis of amphetamines and methamphetamines in urine samples for clinical pharmacology research, bioavailability studies, and forensic toxicology investigations since it is straightforward, sensitive and selective.

CONCLUSIONS

The objective of the current research is to develop an LC-MS method for the analysis and identification of amphetamine and methamphetamine using caffeine as an internal standard. Ultimately, this will allow for the determination of the number of drugs present in urine samples whose constituents are unknown. LLE was favored over other methods since it could be relied upon and did not call for the use of any specialized equipment in the processing of samples. Because of the basic character of the medicines that were used in this experiment, a liquid-to-liquid extraction using dichloromethane as the extracting solvent was carried out on urine samples that had a pH of 12. The UV-Vis spectrophotometer was used in order to arrive at the conclusion that 210 nm should be utilized as the wavelength for the detector.

Authors Contribution

Conceptualization: AZ

Methodology: IUH, FK

Formal analysis: JR, MN

Writing-review and editing: SM, AH, MH, MN, WUK, OUK

All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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