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Research Article

VALIDATED HIGH PERFORMANCE LIQUID CHROMATOGRAPHY METHOD FOR ANALYSIS OF METHAMPHETAMINE IN HUMAN URINE USING LIQUID-LIQUID EXTRACTION

ABU BAKAR RUZILAWATI, HALIJAH MIRAN

Department of Pharmacology, School of Medical Sciences, Universiti Sains Malaysia, Kelantan, Malaysia. Email: tieimran@yahoo.com

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ABSTRACT

Objective: Methamphetamine (also known as pil kuda or syabu) is one of the illicit drugs, which are common among students and teenagers. In Malaysia, methamphetamine was found to be the most commonly abused amphetamine-type stimulants compared with other types of amphetamines. Therefore, fast and reliable detection of these drugs is important in toxicology and doping control. Many extraction methods such as solid phase (SPE) and solid phase micro extraction (SPME) have been used for extraction of methamphetamine in the biological fluid. However, these methods are expensive and may not be available in many laboratories. The objective of this study was to develop an effective liquid-liquid extraction method for methamphetamine in human urine.

Methods: Optimization of the extraction method was done by varying the types of solvents used to extract the drug from the urine. Percentage recovery of methamphetamine extraction was investigated using a variety of extracting solvents (ethyl acetate, dichloromethane, diethyl ether, and chloroform) and solvents system (chloroform:isopropanol [9:1] and chloroform:ethyl acetate:ethanol [3:1:1]). The analysis of the extracted drug was done by high performance liquid chromatography (HPLC) equipped with fluorescence detector. Optimization of chromatographic conditions was also done by varying the pHs of mobile phase and the composition of the mobile phase.

Results: The extracting solvent system that consists of chloroform:ethyl acetate:ethanol (3:1:1) gave the highest recovery, which is 87%. The optimized HPLC condition used for the analysis was a mixture of acetonitrile and deionized water at pH 2.4 in the ratio of 15:85 v/v with a flow rate of 0.6 mL/minute.

Conclusion: This study shows that liquid-liquid extraction method is reliable and cheaper alternative to SPE and SPME.

Keywords: Methamphetamine, Liquid-liquid extraction, High performance liquid chromatography.

INTRODUCTION

Methamphetamine (Fig. 1) or methylated amphetamine belongs to a group of drugs known as amphetamine-type stimulants (ATS) which has almost similar molecular structure to ephedrine compound extracted from plant of *Ephedrae equistina* [1]. ATS drugs consist of ecstasy group such as methylenedioxymethamphetamine, methylenedioxyamphetamine and methylenedioxyethylamphetamine, amphetamine and methamphetamine (in Malaysia also known as pil kuda or syabu).

Methamphetamines are a drug that stimulate central nervous system, sympathomimetic and act as appetite suppressant [2]. Methamphetamine increases dopamine, serotonin and norepinephrine. Dopamine stimulates locomotor effect thus increase capability of being active for longer hours. Serotonin responsible for delusion and psychosis. Early effects following methamphetamines intakes are wakefulness, increased physical activities, decreased appetite and euphoria.

According to Statistic Agensi Dadah Kebangsaan (2009-2013) methamphetamine was found to be the most commonly abused ATS among teenagers and students compared with other types of ATS in Malaysia [3]. Therefore, fast and reliable detection of these drugs is important in toxicology and doping control.

Many extraction methods such as solid phase (SPE) and solid phase micro extraction (SPME) have been used for extraction of methamphetamine in the biological fluid [4,5]. However, these methods are expensive and may not be available in many laboratories.

Several analytical methods are available for the determination of methamphetamine in biological fluids, including gas chromatography mass spectrometer, tandem mass spectrometry (MS/MS) (high-performance liquid chromatography [HPLC]) and liquid chromatography-MS/MS [6-8].

The purpose of this study is to develop an effective liquid-liquid extraction method for methamphetamine in human urine. We choose HPLC with a fluorescence detector for our study since this type of detector is cheaper and available in our laboratory.

METHODS

Chemicals and reagents

Methamphetamine pure standard (1 mg/mL in methanol) and the internal standard, phentermine were purchased from Sigma Aldrich. All chemicals were of analytical purity grade and were purchased from Merck (Darmstadt, Germany).

Instrumentations

The chromatographic waters (Milford, MA, USA) was waters 2695 separation module, which consists of a pump, degasser, autosampler, thermostat operating at 37°C and waters 2475 multi fluorescence detector. Empower software (waters) was used for data acquisition and processing. A reversed-phase acentis express C18 column (150 mm×4.6 mm, 2.7 µm)

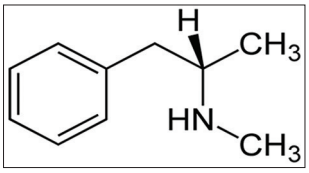

Fig. 1: Structure of methamphetamine

equipped with a guard column (2.7 µm, 5 mm×4.6 mm) was used. The eluted analytes were detected by fluorescence detector at emission, λ_{em}=340 nm and excitation, λ_{ec}=210 nm.

Drug standard preparation

Methamphetamine standard stock solution (1 mg/mL in methanol) was diluted to 5 ug/mL in HPLC grade methanol. Phentermine stock solution was also prepared at 1 mg/mL in methanol. Drug standards were stored in a refrigerator in an amber vial and dilutions were freshly prepared before injecting into HPLC.

Optimization of extraction methods

To develop a single step liquid-liquid extraction procedure with good recoveries, a range of extraction solvents such as dichloromethane, ethylacetate, chloroform, diethyl ether, combination of chloroform:ethyl acetate:ethanol (3:1:1) and combination of chloroform:isopropanol (9:1) were investigated. The percentage recoveries of each solvent were calculated. The best solvent that gave the highest percentage recovery was selected for further investigation on the effect of pH on extraction efficiencies.

Optimization of chromatographic conditions

Optimization of chromatographic conditions was done by varying the pHs of mobile phase and composition of the mobile phase. 200 µl from stock solutions of morphine and internal standard, codeine (5 µg/mL) were injected each time.

We followed Snyder *et al.* [9] recommendation of allowing the column to equilibrate with at least 20 column volumes (approximately 20 minutes) of the new solvent with each change of mobile phase.

RESULTS AND DISCUSSIONS

Several procedures have been published in the literature regarding extraction of the drugs from biological samples. Different types of biological samples have been used for drug analysis including blood, hair, meconium fluids, nails and saliva [10]. In general, urine is the most preferred sample for comprehensive drug screening since it is easy to get and obtained in large volume compared with other biological samples.

According to do Nascimento *et al.* [11], extractions of drugs from biological fluids are usually the most difficult step in any analysis due to the presence of interferences, which need to be removed without causing significant analyte loss. Thus, the sample cleanup technique should be optimized in order to get minimum interference from endogenous impurities [12]. Traditional extraction methods have relied upon protein precipitation agents to remove protein prior to further sample processing [13]. Other methods used for extractions are liquid-liquid, SPE extraction methods and SPME. The use of solid-phase extraction SPE and SPME have been widely used for the toxicological analysis of drugs [5,14,15].

SPE and SPME technique has many benefits such as quick sample processing and reduced analyst exposure to organic solvents [16] as well as avoidance of emulsion creation and the production of cleaner extracts [17]. However, SPE and SPME are expensive and may not be available in many laboratories [14,15]. Liquid-liquid extraction method is a cheaper alternative to SPE and SPME.

In our study, the liquid-liquid extraction method was selected to be used before HPLC analysis as it is a cheaper and easier alternative when compared SPE and SPME method.

In liquid-liquid extraction, a sample is partitioned between two different phases; an organic and an aqueous phase [18]. The solute or drug will transfer out of the aqueous phase (blood or urine) to an organic phase. The organic phase is then evaporated off and the compound or drug can be recovered in a relatively pure state (free from aqueous salts).

In this experiment, a single step liquid-liquid extraction was chosen. To develop this method with good recoveries, a large range of extraction solvents such as dichloromethane, ethylacetate, chloroform, diethyl ether, combination of chloroform:ethyl acetate:ethanol (3:1:1) and combination of chloroform:isopropanol (9:1) were investigated. The absolute recoveries of methamphetamine after a single extraction from urine using these organic solvents were >80% except when combination of chloroform:ethylacetate:methanol (3:1:1) were used where the absolute recovery was 87% (Table 1). Therefore, combination of chloroform:ethylacetate:methanol (3:1:1) were selected to be used in our liquid-liquid extraction method. The combinations of these extracting solvents will facilitate the transfer of the organic layer before drying and produces less noisy chromatograms.

According to Ruzilawati *et al*. [19] the successful analysis of analytes in biological fluids using HPLC relies on good optimization of chromatographic's conditions such as chromatographic separations, the choice of an ideal internal standard and sample preparation.

In this study, to obtain satisfactory separation of methamphetamine and its internal standard phentermine by HPLC, various pH and composition of mobile phase were investigated before proceed with analyzing samples.

In selecting the appropriate mobile phase, several combinations of buffer and organic phases were initially tested. It was found that the mixture of acetonitrile-deionized water (15:85, v/v) produced a good peak for methamphetamine, which was well separated from both its internal standard and the solvent fronts. According to Hubert *et al*. [20] acetonitrile provides good stability and gives lower operating pressures. It also absorbs at lower wavelength as well as is transparent to the wavelength of detection [9]. This solvent has low viscosity, thus providing sharper chromatogram peaks [21]. Silica gel that coats column is also less likely to dissolve in acetonitrile [9]. According to Kromidas [21], acetonitrile/water mixtures have lower back pressure in comparison to methanol/water mixtures. This gives advantages of less wear and tear on seals and columns [21]. Another feature of acetonitrile is it is more toxic than methanol thus hindering microbiological growth in the equipment such as in the column [22].

In this study, mobile phase pHs between 2.5 and 6.5 were tested because silica-based particles are unstable at low pH (pH <2) [23]. The pH of deionized water was adjusted since a gradient elution was used. The best area counts for both methamphetamine and phentermine with the least band tailing were seen at pH 2.4 and, therefore, this pH was selected. Conditions that result in band tailing should be avoided because it generally results in inferior separations and reduced precision [24]. According to Williams [25] band tailing, arises from a number of sources, for example, wrong solvent for sample and inadequate buffering.

The most satisfactory HPLC result was obtained when a mixture of acetonitrile and acidified deionized water with the ratio of 15:85 (v/v) with pH 2.4 were used as mobile phase. The separation completed in 10 minutes using a fluorescence detector with excitation and emission at λ_{ec}=210 nm and λ_{em}=340 nm. Injected sample volume was 20 ul with a flow rate of 0.6 mL/minute which completed in10 minutes. Fig. 2 shows chromatogram of methamphetamine (4.019 minutes) and internal standard, phentermine (4.521 minutes) extracted using a combination of chloroform:ethylacetate:methanol (3:1:1) as extracting solvents.

Table 1: Recovery of methamphetamine from spiked urine sample by liquid-liquid extraction using various solvents

Extracting solvent	Mean recovery (%)
Ethylacetate	No peaks seen
Dichloromethane	No peaks seen
Diethyl ether	No peaks seen
Chloroform:isopropanol (9:1)	78%
Chloroform:ethylacetate:methanol (3:1:1)	87%
Chloroform	No peaks seen

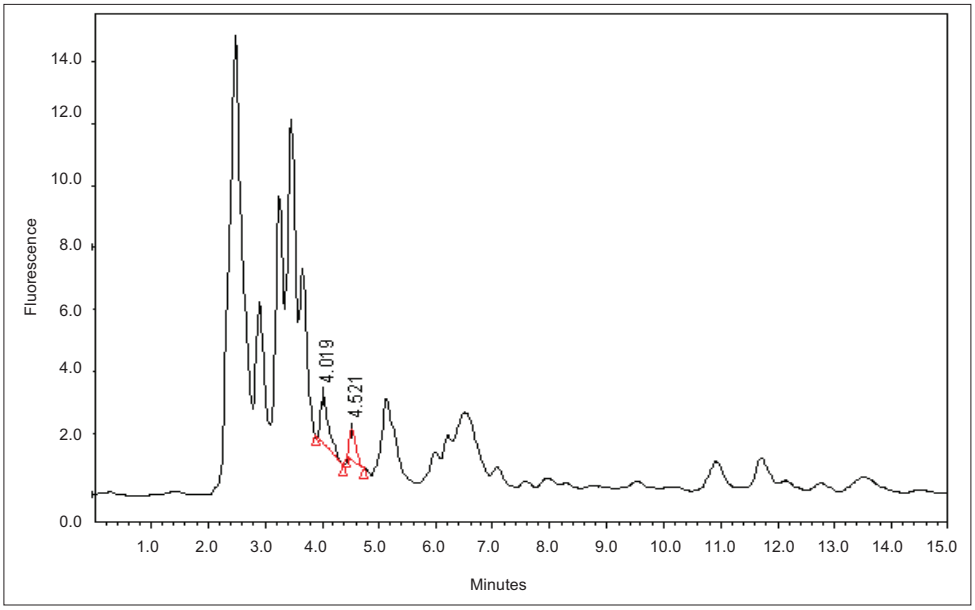

Fig. 2: Chromatogram of methamphetamine (4.019 minutes) and internal standard, phentermine (4.521 minutes) using mixture of chloroform:ethyl acetate:ethanol (3:1:1) as extracting solvents

CONCLUSION

A simple and fast liquid-liquid extraction method and reliable HPLC method with ultra-violet detection for the determination of morphine in human urine has been developed. This method can be a useful method for analyzing the urine of suspected abusers of amphetamine in toxicology analysis and doping control.

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