# Validation of a Simple Extraction Procedure for Stimulants Identification from Human Plasma

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## Abstract

In this study, a novel LC-MS/MS (Liquid Chromatography-Tandem Mass Spectrometry) method was designed using a simple extraction procedure that was scientifically developed to capture the most relevant stimulants, methamphetamine, MDA (3,4-Methylenedioxyamphetamine), MDMA (3,4-Methylenedioxy-methamphetamine), and MDEA (Methyl diethanolamine). The LC-MS/MS method was validated using US FDA (United States Food and Drug Administration) guidelines, and all validation requirements were satisfactory. This protein precipitation method was specifically developed for handling large numbers of samples with minimum cost and volume of sample. The developed method was accurate, precise, and reproducible for quantification of stimulant from human plasma. This pilot study enrolled 100 drug addicts to evaluate stimulant testing from plasma. Our findings found 47 cases with positive methamphetamine. This method may be valuable for stimulant blood screening for a large and varied population because of its efficiency and economical aspects.

***Key words :*** *Stimulant, LC-MS/MS, human plasma, protein precipitation*

## Introduction

Stimulant covers many drugs including those that increase activity of the body and central nervous system, through various mechanism. Stimulant includes amphetamine, metamphetamine, caffeine, ephedrine, MDA, MDMA, MDPV, mephedrone, cocaine, nicotine, phenylpropanolamine, propylhexedrine, and pseudoephedrine. Metamphetamine, MDEA, *MDA, and MDMA were studied in this research.* Stimulants are used to treat sleep disorders, obesity, asthma, mood disorders, nasal congestion, impulse control disorders, and as anesthetics. Amphetamine colorless volatile liquid, has a burning taste and amine odor. It as being very slightly soluble in water, but highly soluble in ethanol and chloroform, with a melting point of 25 °C, and boiling point 200-203°C.[1, 2]

Stimulants increase the presence neurotransmitter dopamine, noradrenaline, and serotonin. They usually block the reuptake or stimulate the efflux of neurotransmitter resulting in increased activity the nerve. Serotonin play a role in mood, appetite, thermoregulation, emotions, sleep, arousal, cognitive, autonomic functions, and pain regulatory systems. The elimination half-life is 6-12 hours. Both hepatic and renal are important organ to eliminate their metabolites from the body (Table 1).[2]

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**Table 1: Pharmacokinetic profile of stimulants**

| No | Profile | MDMA/MDA/ MDEA | Amphetamine | Methamphetamine |
|---|---|---|---|---|
| 1 | Maximal concentration in the blood stream after ingestion | Between 1.5 and 3 hours. | Between 3 and 7 hours. Well absorbed from the gut. | Between 3.13 and 6.3 hours. Well absorbed from the gut. |
| 2. | Target organ in distribution | Most part of the body, especially brain. | | |
| 3. | Duration of action | 4-6 hours | 3-6 hours | 10-20 hours |
| 4. | Metabolism | In liver use CYP450, CYP2D6, CYP3A4, and Catechol-O-methyltransferase | In liver use CYP2D6, glycine N-acyltransferase, dopamine β-hydroxylase, butyrate-CoA ligase, and flavin-containing monooxygenase 3 | |
| 5. | Elimination | Via kidney, about 20% is excreted unchanged in the urine. | Via kidney, about 30–40% of the drug unchanged in the urine. | Via kidney, about 30–54% of the drug unchanged in the urine. |

Source : (2), (3)

Stimulant overdose symptoms and signs are rigid, jerking limbs, seizure, loss of coordination, clumsiness, muscle weakness, slurred or difficult speech, in and out consciousness, fast pulse, chest pain, irregular breathing, cognitive difficulties (confusion, poor judgment, fainting), and psychological distress (anxiety, paranoia, confusion, panicking, hallucinations or extremely agitated).[4]

Stimulants are frequently measured in urine or blood as screening for sports, employment, poisoning diagnostics, and medicolegal investigation with immunoassay or chromatography techniques (Table 2). The MDA concentration in the blood or urine of a person who taking MDMA is less than 10% of parent drug.[3]

**Table 2: Analysis of stimulants in human biological sample using LC-MS/MS**

| No | Analyte | Sensitivity | Sample | Preparation | References |
|---|---|---|---|---|---|
| 1 | Stimulants | LOQ : 0.025-0.1 ng/mL | Urine | SPE | (5) |
| 2 | Stimulants | LOQ : 2.03–2.48 ng/mL | Plasma | Protein Precipitation | (6) |
| 3 | MDMA, MDA | LOQ : 100 ng/mL | Urine | SPE | (1) |
| 4 | Stimulants | LOQ : 2.5 ug/L | Blood, Urine | SPE | (7) |

## Materials and methods

### Materials and standards

The Metamphetamine, MDEA, MDA, MDMA, and IS (Methamphetamine-d5, MDA-d5, and MDMA-d5) standard was purchased from Sigma-Aldrich (St. Louis, MO, USA). All solvents and reagents used were of HPLC grade and purchased from Merck (Darmstadt, Germany). Control plasma samples were obtained from the UMMC blood bank Malaysia**.**

### Samples

A total of 100 blood samples were obtained from drug addiction that consenting to participation in the study. Demographic details such as gender, age, residential area, and ethnicity data were recorded. The inclusion criteria were 18 years of age or older, fully conscious, physically healthy, and no serious current psychiatric symptoms (i.e. psychotic episode). The exclusion criteria were refusal to participate, requiring advanced medical attention for a serious illness, and requiring psychiatric care for psychiatric symptoms. Institutional approval for the analysis of human samples was obtained from the Ethical Committee of University Malaya Medical Centre. The collection sample method used the method was published before.[8]

### Extraction of plasma

Sample preparation was carried out using a protein precipitation extraction method**.** The frozen plasma was thawed at room temperature (25 ± 1°C). The thawed plasma was vortexed to ensure the sample was homogenous. To each 100 µL plasma sample, 50 µL of internal standard (IS) (containing 120 ng/mL of IS) was added, followed by the addition of 250 µL of acetonitrile (ACN). The mixture was vortexed, shaken for 5 s, and centrifuged for 2 min at 14800 rpm. The supernatant was filtered with a 0.2 µm syringe filter, then transferred to new a vial. Eight microliters were then injected into the LC-MS/MS system.

### Liquid chromatography apparatus and conditions

In this research, the LC system consisted of an LC-20AD XR UFLC system with a SIL-HT automatic sample injector (Shimadzu, Kyoto Japan) were used. The analytical column used was a Agilent, Eclipse Plus Phenyl-Hexyl (150 mm length x 2.1 mm ID, particle size 5µm ). Column temperature was 40°C with total running time 10 min. Mobile phase used were 10 mM ammonium formate in water (pH 6.6) in pump A and 0.1% FA (in ACN) in pump B. The flow rate was set at 0.3 mL/min and a gradient elution was used at room temperature. The gradient program began with 5% B, then ramped to 40% B at 3.00 min, and held until 4.00 min. The gradient was ramped to 100% B at 6.00 min and held until 8.00 min.The gradient then returned to 5% B at 8.01 min and this condition was held for a further 10.00 min. Sample injection volume was 8 µL.

### Mass spectrometry parameters

A Linear Ion Trap Quadrupole LC-MS/MS Spectrometer, QTRAP 5500, fitted with an ESI probe, and operated in the negative ionization mode was used to perform mass spectral analysis. The LC-MS/MS system was controlled by the Analyst software, version 1.6.3 (Applied Biosystems). Nitrogen was used as the nebulizer, auxiliary, collision, and curtain gas. Analytes were then quantified by multiple reactions monitoring (MRM). The optimal conditions were as follows: ion source temperature of 450°C, ion spray voltage of 5500 V, curtain gas of 20.0 psi, collision gas of medium, ion source gas 1 of 35.0 psi, and ion source gas 2 of 35.0 psi. Analyst 1.6.3 software was used for system control and data quantification.

## Results

### Linearity, sensitivity, and specificity

No significant interfering peaks were observed from the retention time corresponding to stimulants and the internal standards (Fig. 1-2). Seven calibration points were used to evaluate the linearity of the standard calibration curve. The standard curve was linear with 1/X as weighing factor and reached good linearity ($r > 0.997$). The signal to noise ratio which was greater than 10. The LOD for MDMA and methamphetamine was established at 0.25 ng/mL; MDA and MDEA was 1.25 ng/mL.

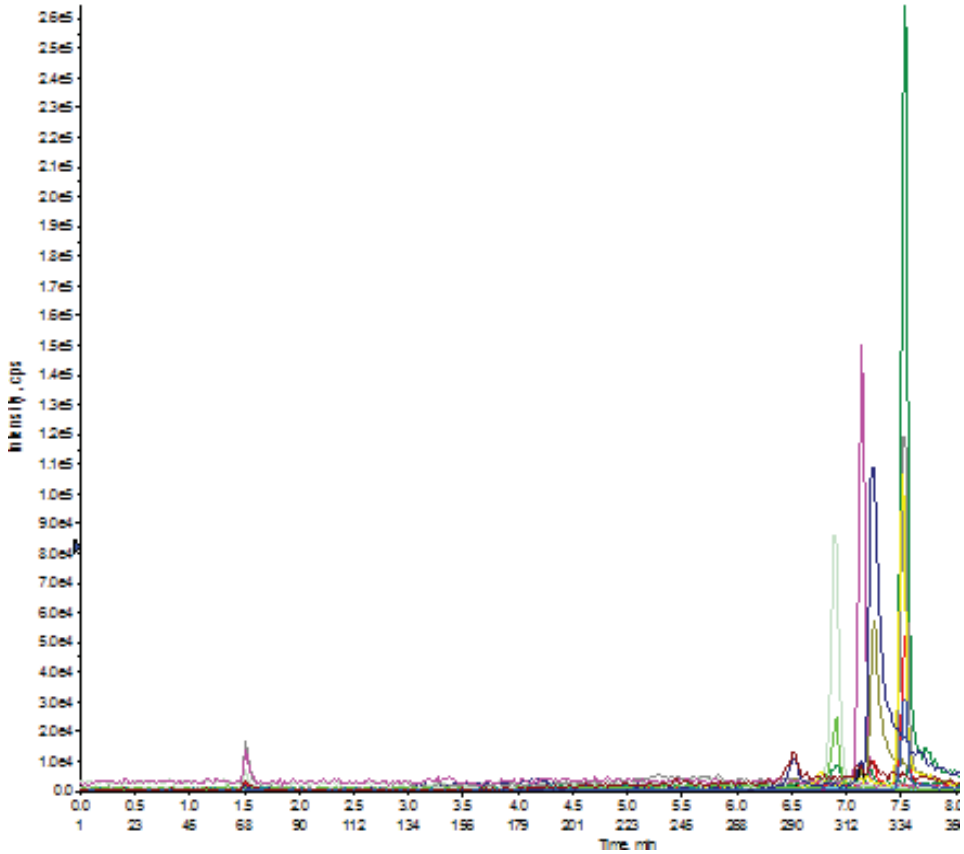

**Fig. 1. Representative MRM chromatogram of blank.**

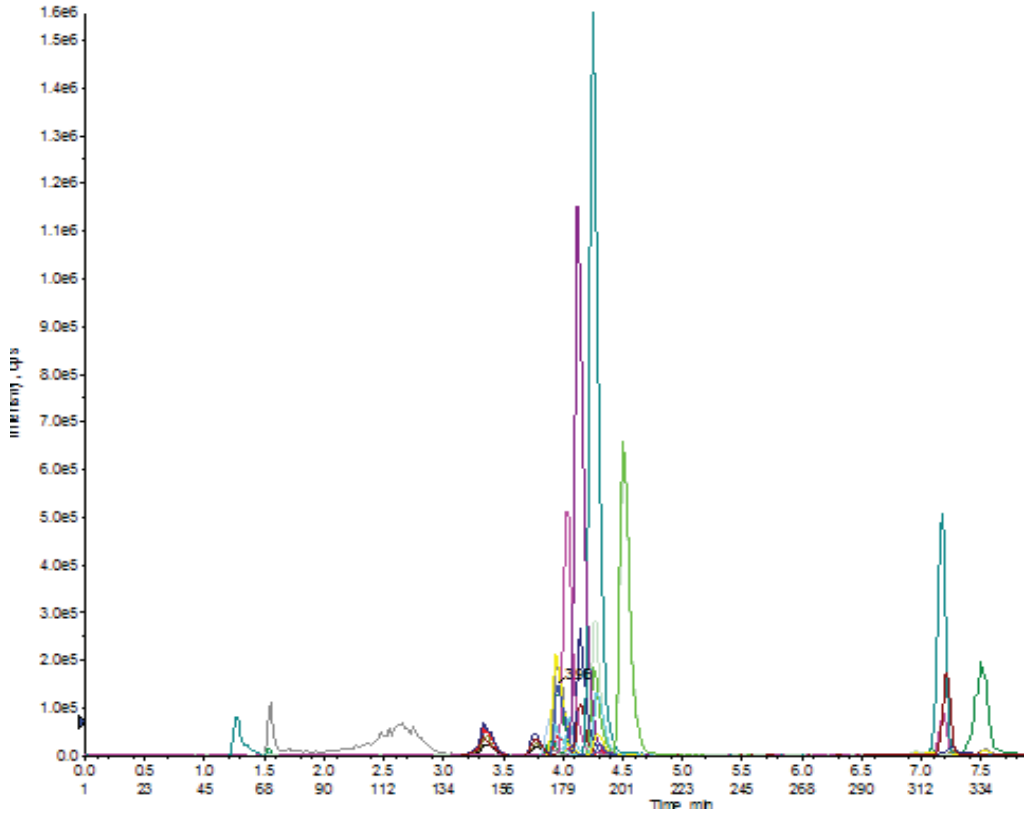

**Fig. 2. Representative MRM chromatogram of stimulants in human plasma (QCL:1.5e6)**

Precision and accuracy

Based on the mean percentage of coefficient of variation (%CV) for five quality control samples, the intra-day precision of plasma sample ranged from 0.41% to 9.93%. When assessed by means of the five quality control samples the accuracy for LLOQ intra-day plasma sample ranged from 98.16% to 118.4%, then for others ranged from 89.22% to 114%. The inter-day precision and accuracy were 7.17% to 9.97% and 95.4% to 113.12%. The results from this data clearly showed that the method that was developed has good accuracy, precision, and reproducibility for the quantification of stimulants from human plasma.

**Recovery**

The recovery of stimulants was tested at 0.5 and 5 ng/mL (Table 8). These results indicated that the extraction efficiency of stimulants using protein precipitation was quite good.

**Table 3: Recoveries for DoA in human plasma**

| Compound | 0.5 ppb | 5 ppb |
|---|---|---|
| MDA | 100% | 91.9% |
| MDMA | 97.5% | 96.6% |
| Methamphetamine | 96.8% | 89.3% |
| MDEA | 92.4% | 88.1% |

**Stability testing**

The stimulants precision ranged from 0.41% to 9.72% and accuracy ranged from 88.22% to 114% for freeze and thaw stability in plasma. The results indicated that the stimulants was stable in plasma for two cycles when stored at -20ºC and thawed to room temperature. The precision and accuracy for stimulants bench top 4 hours stability in plasma ranged from 1.35% to 9.72% and from 95.08% to 114% respectively. The results indicated that the stimulants was stable in plasma at room temperature up to 4 hours when put on the bench. The precision and accuracy for stimulants autosampler stability in plasma ranged from 1.75% to 9.51% and from 98.54% to 114.2% respectively. The stimulants in plasma sample could be analyzed over 24 h in an autosampler at 20±1$^0$C with satisfactory precision and accuracy. The precision and accuracy for stimulants long term (2 months) stability in plasma ranged from 1.59% to 8.87% and from 91.16% to 108.2% respectively.

## Discussion

**Analysis of plasma samples**

This method was applied to the screening of stimulants levels in human blood plasma that were taken from 100 drug addicts of Malaysia (Table 4). Mostly participant from Kuala Lumpur and Selangor. Research subjects were 46.03±10.31 years old in average, with the youngest was 21 and the oldest was 72 years old. The mean of height and weight was 164.42±7.48 cm and 61.43±13.03 kg respectively.

**Table 4. Baseline characteristics of participants.**

| **Variable** | **N (%)** | **Variable** | N (%) |
|---|---|---|---|
| Gender | | Job Scope | |
| -Male | 95 (95) | -Jobless | 26(26) |
| -Female | 5 (5) | -Private sector employee | 74(74) |
| Age Group | | Types of Food | |
| -18-30 yo | 7 (7) | -Boiled | 16 (16) |
| -31-40 yo | 26 (26) | -Fried | 84 (84) |
| -41-50 yo | 34 (34) | -Raw | 0 (0) |
| -51-60 yo<br>-61 yo and abobe | 24 (24)<br>9 (9) | Source of Food<br>-Home cooking | 68 (68) |
| BMI | | -Dining out | 23 (23) |

**Table 4. Baseline characteristics of participants.**

| | | | |
|---|---|---|---|
| -Under weight | 19 (19) | -Take away | 9 (9) |
| -Normal weight | 57 (57) | Smoking | |
| -Pre obesity | 12 (12) | -Yes | 85 (85) |
| -Obesity class 1 | 12 (12) | -No | 15 (15) |
| Hometown | | Source of Drinking | |
| -West Malaysia | 149 (149) | -Treated water | 72 (72) |
| -South Malaysia | 1(1) | -Bottle water | 28(28) |
| Race | | | |
| -Malaysian | 80 (80) | | |
| -Chinese | 9 (9) | | |
| -Indian | 11 (11) | | |
| | | | |

In this study the majority of drug addicts were men. More addicts are men because they are braver and more easily influenced by the environment than women.. There are addicts who started taking drugs because of curiosity to try it. There are also those who use drugs to get recognition of their social status, also to feel the same experience with their friends. The results of this study are consistent with several other studies that show more male addicts than female. A study in Pakistan involving 150 respondents, was 100% male.[9,10]

In this study most addicts were of working age. The possible cause is the heavy workload that exists and life style factor. Heavy workload, will be a heavy stressor too, so there are workers who are looking for pleasure by consuming drugs. According to combined 2008 to 2012 National Survey on Drug Use and Health (NSDUH), 113.1 million persons of US Population are full-time workers. In the other hand, some of the addicts don't even have a job. This is consistent with NSDUH, 13 million addicts in the USA do not work. There are some of the addicts who lost their jobs, because of using drugs.[11]

Drug abusers tend to lose weight. This is because immunity in general decreases, so it often suffers from illness both mild illness and long-term pain, such as HIV and hepatitis. HIV and hepatitis are diseases that can be transmitted through sharing needles and free sex between drug addicts. In 2007, the number of HIV in Russia among drug addicts reached 1.7 million, while the injecting drug user (IDU) with HIV reached 1.2 million. In 2002, the number of HIV in the USA among drug addicts reached 1.9 million, while the injecting drug user (IDU) with HIV reached 1.7 million. In 2005, the number of HIV in China among drug addicts reached 2.3 million, while the injecting drug user (IDU) with HIV reached 2.1 million.[12]

Chronic diseases will stimulate the body's immunity to be more active, and to stimulate metabolism faster. This can reduce the amount of fat in the body, if it continues continuously the addict will become underweight. In this study, the majority of addicts had a normal body mass index. The second place is underweight with some suffering from chronic diseases.[13]

Almost all addicts in this study were active smokers. This is consistent with studies in Pakistan, that the majority of addicts are active smokers. In drug addicts, the number of cigarettes smoked was affected by the type of drug consumed. Heroin users, had the highest cigarette consumption compared to other drugs.[14] This can cause various respiratory illnesses. In addition, smoking will also put dozens of poisons into the body.[10]

From blood screening, we detected 47 cases with positive methamphetamine. Methamphetamine use, can be via oral, intranasal, smoking, and intravenous. The largest intravenous bioavailability, which reached 100%, while for smoking and oral about 67% and 79% intra nasal. Methamphetamine is metabolized in the liver by a process: N-demethylation to produce amphetamine, (ii) aromatic hydroxylation to produce primarily 4-hydroxymethamphetamine; and (iii) β-hydroxylation to produce norephedrine. Polymorphic cytochrome P450 2D6 causes different metabolisms in methamphetamine. Methamphetamine in the body has a different detection time, in the blood 24-48 hours, in saliva 24 hours, in urine up to 87 hours after taking 10 mg of methamphetamine tablets. Around 70% of a methamphetamine dose is excreted in the urine (within 24 hours) : 30-50% as methamphetamine, 10% as amphetamine, and up to 15% as 4-hydroxymethamphetamine. Acute effects will appear for more than 8 hours.[15]

## Conclusions

This study successfully employed an inexpensive, rapid, and simple procedure for stimulants extraction and subsequent detection from human plasma by LC-MS/MS. The plasma extraction consisted of a simple protein precipitation method that consumes a low volume of organic solvent and trace volume of sample (100 µl). The method was suitable for identifying and effectively separating stimulants with good precision and accuracy. This pilot study enrolled 100 drug addicts to evaluate stimulants testing from plasma. Our findings found 47 cases with positive methamphetamine. Determination of stimulants human plasma concentrations from the subjects could be satisfactorily performed by this proposed method.

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