

Development and Validation of GC-MS Method for Cocaine in Human Urine

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Abstract

This paper describes a gas chromatography-mass spectrophotometry (GC-MS) method with selected-ion monitoring (SIM) for the measurement of urine cocaine levels. Cocaine and codeine (internal standard, IS) were extracted from human urine with a mixture of diethylether and ethylacetate at basic pH with liquid-liquid extraction. Calibration curve was linear over the concentration range 50-2000 ng mL⁻¹. Intra- and inter-day precision values for cocaine in human urine were less than 6.9, and accuracy (relative error) was better than 6.8%. The analytical recovery of cocaine from human urine averaged 92.9%. The limits of detection (LOD) and quantification (LOQ) of cocaine were 15 and 50 ng mL⁻¹, respectively. Stability studies showed that cocaine were stable in human urine after 8 h incubation at room temperature or after 1 week storage at -20 °C with three freeze-thaw cycles.

Introduction

Cocaine (Figure 1) is one of the most abused controlled dangerous substance drugs after amobarbital, secobarbital and methadone.

Cocaine is a highly active stimulant, and few programs report significant numbers of cocaine-positive urine specimens because cocaine undergoes extensive biotransformation leaving minimal quantities of the parent drug present [1,2].

According to the National Institute on Drug Abuse at the National Institutes of Health, a 1996 study showed that "crack" cocaine continues to dominate the nation's illicit drug problem [3]. In 1996, 1.75 million people were using cocaine, in both the acid and base form, in the United States. Cocaine undergoes reaction in the body forming a number of major metabolites such as benzoylecgonine and ecgonine methyl ester [4,5] that are then excreted in the urine, sweat, saliva and faeces. When cocaine is ingested with alcohol, another metabolite, cocaethylene, is produced in the liver causing intensified euphoric effects [6].

Cocaine and its metabolites can be measured by several analytical techniques including immunoassay [7], HPLC [8-12], gas chromatography [13-16], and GC-MS [17-23]. Generally, these methods focus on the determination of cocaine, benzoylecgonine or ecgonine methyl ester.

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Immunoassay technique is not always specific. Because it can detect both of the free and conjugated cocaine. HPLC coupled with an UV detector has been widely adopted to determine cocaine levels in biological samples, but normally needs postcolumn derivatization to increase its sensitivity during analysis. The retention times used for identification in HPLC are also not very reproducible. Mass spectrometry offers the structural information of cocaine and high sensitivity, as well as specificity in detection. Therefore, GC-MS provides an alternative method for cocaine analysis.

Solid-phase extraction systems have been reported for the rapid and efficient removal of numerous cocaine and metabolites [24]. The ability of these copolymeric bonded phase columns to extract both basic and aniphoteric cocaine metabolites allows the design of an assay for the simultaneous determination of multiple metabolites of cocaine. These methods are also the most comprehensive method which can extract cocaine in a single extraction procedure.

On extensive survey of literature, no liquid-liquid phase extraction method is reported till date for determination of cocaine by GC-MS in human urine. Therefore, in this study, liquid-liquid phase extraction system has been used. Then, we report a GC-MS with SIM method for determination of cocaine in human urine using internal standard methodology. The developed method was validated by using linearity, stability, precision, accuracy and sensitivity parameters according to International Conference on Harmonization (ICH) guidelines [25]. The advantages of present method include simple and single step extraction procedure using inexpensive chemicals and short run time.

Material and Methods

1. Chemicals and reagents

Cocaine and codeine as Internal Standard (IS) were obtained from Criminal Police Laboratory (Erzurum, Turkey). Butyl chloride, diethylether, ethylacetate, hexane, dichloromethane, acetonitrile and butanol were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2. GC-MS system

Chromatographic analysis was carried out on an Agilent 6890N gas chromatography system equipped with 5973 series mass selective detector, 7673 series autosampler and chemstation (Agilent Technologies, Palo Alto, CA). HP-5 MS column with 0.25 μm film thickness (30 m \times 0.25 mm I.D., USA) was used for separation. Splitless injection was used and the carrier gas was helium at a flow rate of 1 mL min⁻¹. The injector and detector temperatures were 250 °C. The MS detector parameters were transfer line temperature 290 °C, solvent delay 3 min and electron energy 70 eV.

3. Preparation of stock and standard solutions

The stock standard solutions of cocaine and IS were prepared with acetonitrile to a concentration of 5000 ng mL⁻¹ and stored at -20 °C under refrigeration. Standard solutions at 50, 200, 400, 800, 1200, 1600 and 2000 ng mL⁻¹ concentrations of cocaine were prepared by diluting with acetonitrile appropriate volumes of stock solution. Preparation of urine working solutions; a suitable amount of standard cocaine solutions together with 500 ng mL⁻¹ IS was spiked in 1.0 mL urine and then extracted with liquid-liquid extraction method. The quality control (QC) solutions were prepared by adding aliquots of standard working solution of cocaine to a final concentrations of 150, 1000 and 1750 ng mL⁻¹, 0.1 mL of IS (500 ng mL⁻¹) and 1.0 mL urine blank.

4. Extraction procedure

A 1.0 mL blank urine sample was transferred to a 12 mL centrifuge tube together with 0.1 mL IS solution (500 ng mL⁻¹) and 0.5 mL 1 M sodium hydroxide solution. After vortex mixing for 5 second, 4 mL of diethylether and ethylacetate was added (3:1, v/v), the mixture was vortexed for 30 second and then centrifuged at 3000 x g for 3 min. The organic layer was transferred into another tube and evaporated to dryness at room temperature under nitrogen gas. The dry residue was dissolved in 1 mL of acetonitrile. Then 1 μL of aliquot was injected into the GC-MS system. The mass spectra of the cocaine and IS are shown in Figure 2.

5. Method validation

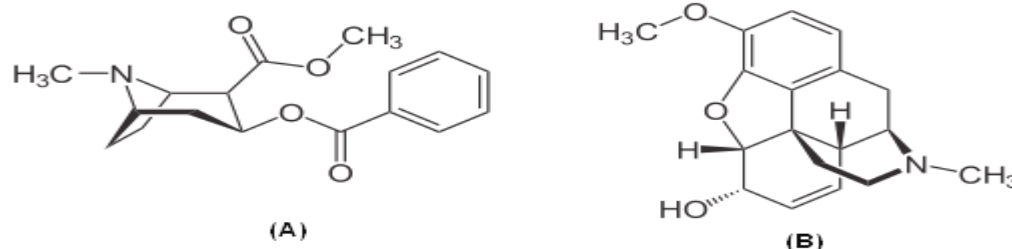
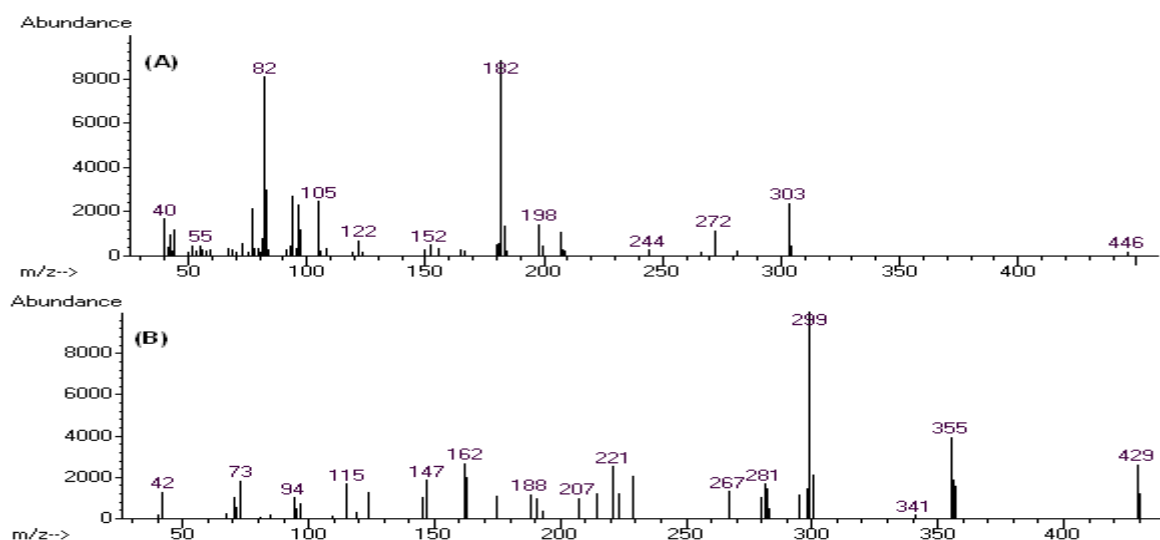
The peak-area ratios (cocaine to IS) were determined in triplicate. The calibration curves were obtained by a

least-squares linear fitting of the peak area ratios versus the amounts of cocaine. Intra- and inter-run precision were assessed from the results of QCs. The mean values and RSD for QCs at three concentration levels were calculated over six validation runs. These values were then used to calculate the intra- and inter-run precision (RSD) by a one-way analysis of variance. The accuracy of the method was determined by calculating the percentage deviation observed in the analysis of QCs and expressed as the relative error (RE). The precision and accuracy of each QC value should not exceed a deviation of 15%, except for the QC samples for the limit of quantification (LOQ) where 20% was acceptable.

Table 1: Linearity of cocaine in human urine.

Linearity (ng mL ⁻¹)	50-2000
Regression Equation ^a	$y=0.0025x+0.2624$
Standard Deviation of Slope	3.87×10^{-4}
Standard Deviation of Intercept	4.85×10^{-3}
Correlation Coefficient	0.998
Standard Deviation of Correlation Coefficient	2.43×10^{-4}
Limit of Detection (ng mL ⁻¹)	15
Limit of Quantification (ng mL ⁻¹)	50

^aBased on three calibration curves, y: peak-area ratio, x: cocaine concentration (ng mL⁻¹)


Figure 1: Structural formula of cocaine (A) and codeine (IS) (B).

Figure 2: MS spectra of cocaine (A) and codeine (IS) (B).

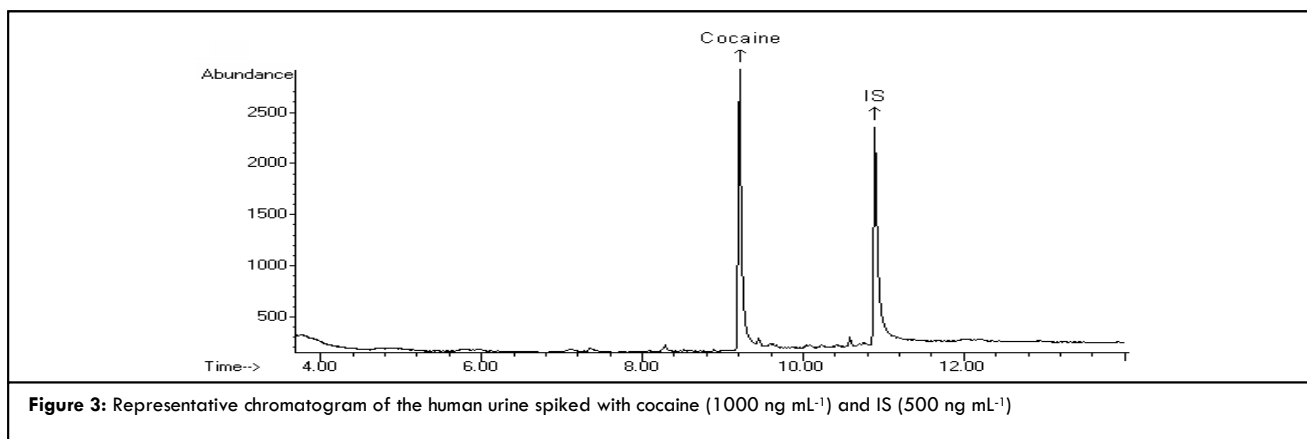


Figure 3: Representative chromatogram of the human urine spiked with cocaine (1000 ng mL⁻¹) and IS (500 ng mL⁻¹)

Results and Discussion

1. Method development and optimization

The method development for the assay of cocaine was based on their chemical properties. In this study, the capillary column coated with 5% phenyl, 95% dimethylpolysiloxane is a good choice for separation of these analytes since they elute as symmetrical peaks at a wide range of concentrations. Different temperature programs were investigated for GC oven. The end of this investigation, the best temperature program was selected for a good separation. The temperature programs of the GC oven were as follows: initial temperature 150 °C, held for 1 min, increased to 220 °C at 20 °C min⁻¹ held for 1 min, and finally to 300 °C at 10 °C min⁻¹ with a final hold of 1 min. The splitless injection mode was chosen. Additionally, preliminary precision and linearity studies performed during the development of the method showed that the 1 µL injection volume was reproducible and the peak response was significant at the analytical concentration chosen.

2. Validation of the method

2.1. Specificity: The specificity criterion tries to demonstrate that the result of the method is not affected by the presence of interferences [26]. The specificity of method was determined by checking the chromatograms obtained from blank urine samples, and no endogenous interferences were encountered (Figure 3). The fragment ions (*m/z* 182 and 299) were used for quantitation of cocaine and IS at SIM mode. The retention time of cocaine and IS in human urine was approximately 9.3 and 10.9 min with good peak shape.

2. Linearity: Calibration curve in human urine was constructed by spiking seven different concentrations of cocaine. The chromatographic responses were found to be linear over an analytical range of 50-2000 ng mL⁻¹. The equation of the calibration curve obtained from seven points was $y=0.0025x+0.2624$ with a correlation coefficient ($r=0.998$). The linear regression equation was calculated by the least squares method using Microsoft Excel® program and summarized in Table 1.

2.3. Precision and accuracy: The precision and accuracy of the method were examined by adding known amounts of cocaine to blank human urine. For intra-day precision and accuracy, six replicate QC samples at each concentration were assayed on the same day. The inter-day precision and accuracy were evaluated on three different days. The intra- and inter-day precisions of the QC samples were satisfactory with RSD less than 6.9% and accuracy with RE within $\pm 6.8\%$ (should be less than 15 according to ICH guidance) [25].

2.4. Sensitivity: The sensitivity was evaluated by the limit of quantification (LOQ), the lowest concentration of the urine spiked with cocaine in the calibration curve. The limit of detection (LOD) was determined as the lowest concentration, which gives a signal-to-noise ratio of 3 for cocaine. Under the experimental conditions, the LOQ value was 50 ng mL⁻¹. Also, the LOD value was 15 ng mL⁻¹.

2.5. Recovery: Recovery was performed to verify the effectiveness of the extraction step and the accuracy of the proposed method. The liquid-liquid extraction was used for the sample preparation in this work. Several solvents (butyl chloride, diethylether, ethylacetate, hexane, dichloromethane, acetonitrile and butanol) were

tested for the extraction. Finally, diethylether and ethylacetate mixture (3:1, v/v) proved to be the most efficient in extracting cocaine from human urine. After extraction procedure, the dry residue was dissolved in 1.0 mL of acetonitrile. The mixture was vigorously shaken and then delayed at room temperature for 10 min. Spiked plasma samples were prepared in three time at all levels (50, 200, 400, 800, 1200, 1600 and 2000 ng mL⁻¹) of the calibration graph of cocaine. The recovery of cocaine was determined by comparing the ratio of the amount of cocaine and IS measured after analysis of spiked urine samples with those found after direct injection of standard solutions at the same concentration levels. The mean extraction recovery of cocaine in human urine was 92.9%. The mean relative recovery for IS at 500 ng mL⁻¹ was 95.2% ($n = 3$).

2.6. Matrix effect: The matrix effect was defined as the direct or indirect alteration or interference in response due to the presence of unintended analytes or other interfering substances in the sample [27]. The matrix effect of cocaine was investigated by comparing amount of cocaine solutions with processed blank samples reconstituted with cocaine solutions. The blank urines used in this study were from three different batches of human blank urine. If the ratio <85% or >115%, a matrix effect was implied. The relative matrix effect of cocaine at three different concentrations (200, 1000 and 2000 ng mL) was less than $\pm 10.8\%$. The results showed that there was no matrix effect of the analytes observed from the matrix of urine in this study.

2.7. Stability: The stability of cocaine in human urine was assessed by analyzing low and high concentration level samples after storage for different times and temperatures. The short-term temperature stability was assessed by analyzing three aliquots of each of the low and high concentration samples (200 and 2000 ng mL) at room temperature for 8 h. Freeze-thaw stability (-20 °C in urine) was checked through three cycles. Samples were stored at -20 °C for 24 h and then thawed unassisted at room temperature. When completed thawed, samples were refrozen for 24 h. Samples were analyzed after three freeze-thaw cycles. The long-term stability was assessed after storage at -

20 °C for 1 week. No significant degradation of cocaine was observed under the tested conditions.

Conclusion

In the present work, a simple and sensitive GC-MS method has been developed for the determination of cocaine in human urine. Also, the method was completely validated by using sensitivity, stability, specificity, linearity, accuracy and precision parameters for determination of cocaine in human urine. The method was found to be linear over an analytical range of 50-2000 ng mL⁻¹. Also, the extraction procedure in this study was simple. No significant interferences and matrix effect caused by endogenous compounds were observed. To our knowledge, this is the first liquid liquid extraction description of cocaine in human urine by GC-MS method in the literature. This is important for forensic purposes. Because this procedure allows to detect cocaine after ingesting an overdose of the drug.

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