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Simultaneous Determination of Heroin and Its Metabolite in Human Plasma by Gas Chromatography-Mass Spectrometry

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ABSTRACT

A simple and sensitive gas chromatography-mass spectrometry (GC-MS) method for determination of heroin and its metabolite (morphine) in human plasma was developed and validated. Medazepam was used as an internal standard (IS). The calibration curves were linear ($r = 0.99$) over heroin and morphine concentrations ranging from 10 to 200 ng mL^{-1} and 7.5-300 ng mL^{-1}, respectively. The method had an accuracy of >95% and intra- and inter-day precision (RSD%) of ≤4.83% and ≤4.68% for heroin and morphine, respectively. The extraction recoveries were found to be 97.63 ± 1.21 and 96.33 ± 1.66% for heroin and morphine, respectively. The limit of quantification (LOQ) using 1 mL human plasma was 10 ng mL^{-1} for heroin and 7.5 ng mL^{-1} for morphine. Stability studies showed that heroin and morphine were stable in human plasma after 8 h incubation at room temperature or after 1 week storage at -20 ^{0}C with three freze-thaw cycles.

Key words: Heroin; Morphine; GC-MS; Liquid-liquid extraction; Validation.

INTRODUCTION

Heroin is a semi-synthetic lipophilic morphine derivative. Two acetyl-groups are coupled to the 3- and 6-carbon site of the phenanthrene ring. Heroin would pass the blood-brain barrier more easily than morphine because of the acetyl-groups. The ester bonds are very unstable in human plasma and heroin is rapidly hydrolysed to 6-monoacetylmorphine and morphine by serum-esterases and liver carboxylesterases[1].

Heroin has a very short estimated half-life between 2 and 5 min. It mainly acts by its more stable agonistic metabolites 6-monoacetylmorphine, morphine and morphine-6-glucuronide[2].

Several methods have been reported for the determination of heroin or morphine including spectrofluorometry[3], radioimmunoassay[4,5], enzyme-multiplied immunoassay[6,7], high performance liquid chromatography (HPLC)[8-10] and gas chromatography-mass spectrometry (GC-MS)[11,12], liquid chromatography tandem mass spectrometry (LC-MS-MS)[13] in pharmaceutical preparations and biological fluids.

Immunoassay technique is not always specific. Because it can detect both of the free and conjugated morphine. HPLC coupled with an electrochemical detector has been widely adopted to determine morphine 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 heroin or morphine and high sensitivity, as well as specificity in detection. Therefore, GC-MS provides an alternative method for heroin and morphine analysis.

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This paper describes a simple and specific GC procedure with MS detection for determining heroin and morphine in human plasma. The developed method was validated by using linearity, stability, precision, accuracy and sensitivity parameters according to International Conference on Harmonization (ICH) guidelines[14].

EXPERIMENTAL

Chemicals and Reagents

Heroin hydrochloride, morphine hydrochlororide and medazepam as internal standard (IS) were obtained from Criminal Police Laboratory (Erzurum, Turkey) (Fig. 1). Ethylacetate, *N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA)* and sodium acetate were purchased from Sigma-Aldrich (St. Louis, MO, USA).

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 ìm film thickness (30 m × 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 ^{0}C. The MS detector parameters were transfer line temperature 290 ^{0}C, solvent delay 3 min and electron energy 70 eV.

Preparation of Stock and Standard Solutions

Stock solutions of heroin and morphine were prepared by dissolving the accurately weighed reference compounds in methanol to give a final concentration of 10 µg mL^{-1} of both. The solutions were then serially diluted with methanol to achieve standard working solutions at concentrations of 10, 25, 50, 100, 150, 175, 200 ng mL^{-1} and 7.5, 15, 25, 50, 100, 200, 300 ng mL^{-1} for heroin and morphine, respectively. Stock solution of IS was prepared in methanol at the concentration of 5 µg mL^{-1}. All the solutions were stored at 4 ^{0}C and were brought to room temperature before use. The quality control (QC) solutions were prepared by adding aliquots of standard working solution of heroin and morphine to final concentrations of 12.5, 75 and 175 ng mL^{-1} and 7.5, 75 and 250 ng mL^{-1} containing 0.1 mL IS (500 ng mL^{-1})

Extraction Procedure

Blood samples were collected into the tubes containing disodium EDTA and centrifuged at 4500 × g for 10 min. A 1 mL of the resultant plasma samples was spiked with 1 mL of heroin or morphine, 0.1 mL of IS and 1 mL sodium acetate solution (pH 5.5) were added. After vortex mixing for 5 s, 4 mL of ethylacetate was added. The mixture was vortexed for 30 s and then centrifuged at 3000×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 100 µL of a mixture of acetonitrile and *MSTFA* (50:50, *v/v*). The mixture was vigorously shaken and then delayed at 60 ^{0}C temperature for 15 min. 1 µL sample was injected into the GC-MS system.

Stability of Plasma Samples

The stock solutions of heroin (10 µg mL^{-1}), morphine (10 µg mL^{-1}) and IS (5 µg mL^{-1}) were prepared in methanol. All solutions were stored at 4 ^{0}C. The stability of heroin and morphine in plasma was evaluated for 1 week at -20 ^{0}C, and for three freeze-thaw cycles. The validation criteria were calculated using commonly accepted statistical procedures.

Method Validation

The peak-area ratios (heroin to medazepam or morphine to medazepam) were determined in triplicate. The calibration curves were obtained by a least-squares linear fitting of the peak

area ratios versus the amounts of heroin or morphine. 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.

RESULTS AND DISCUSSION

Method Development and Optimization

The method development for the assay of heroin and morphine 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

Table 1: Results of regression analysis of the linearity data of heroin and morphine

	Mean ± SD (n=3)	
	Heroin	**Morphine**
Slope	0.2186 ± 0.0264	0.4264 ± 0.0164
Intercept	0.0272 ± 0.0032	0.0364 ± 0.0018
Correlation coefficient (r)	0.9913 ± 0.0004	0.9924 ± 0.0006

SD: Standard deviation of three replicate determinations

Table 2: Intra-day and inter-day precision and accuracy of heroin and morphine in human plasma (n=6)

Sample	Concentration (ng mL^{-1})		%RSD	%RE
	Added	**Found (Mean ± SD)**		
Heroin	12.5	11.86 ± 0.236	1.99	-5.12
Intra-day	75	72.58 ± 1.362	1.88	-3.22
	175	170.26 ± 2.046	1.20	-2.71
Inter-day	12.5	12.13 ± 0.586	4.83	-2.96
	75	79.03 ± 2.624	3.32	5.37
	175	176.02 ± 5.84	3.32	1.36
Morphine	7.5	7.34 ± 0.286	3.89	-2.13
Intra-day	75	70.58 ± 1.532	2.17	-5.89
	250	242.62 ±3.164	1.30	-2.95
Inter-day	7.5	7.31 ± 0.342	4.68	-2.53
	75	71.48 ± 1.823	2.55	-4.69
	250	228.16 ± 7.924	3.47	-8.74

RSD: Standard deviation of six replicate determinations

Accuracy: (%relative error, %RE)= (found-added)/added×100

temperature 150 ^{0}C, held for 1 min, increased to 200 ^{0}C at 12.5 ^{0}C min^{-1} held for 1 min, and finally to 290 ^{0}C at 30 ^{0}C min^{-1} with a final hold of 4 min. The splitless injection mode was chosen. Additionally, preliminary precision and linearity studies performed during the development of the method showed that the 1 mL injection volume was reproducible and the peak response was significant at the analytical concentration chosen.

Validation of the Method

The validation was carried out by establishing specificity, linearity, intra- and inter-day precision, accuracy, recovery and sensitivity parameters according to ICH[14].

Specificity

The specificity of method was determined by checking the chromatograms obtained from blank plasma samples (Fig. 2). The fragment ions (m/z 369, 429 and 242) were used for quantitation of heroin, morphine-di-TMS and IS. The retention times of IS, morphine and heroin were 5.7, 6.9 and 7.5 min, respectively and the total run time of analysis was 8 min.

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No peaks from endogenous compounds that interfered with heroin, morphine-TMS and IS were observed in the plasma samples.

Linearity

Calibration curves for the plasma assay developed with a peak-area ratio (*y*) of heroin or morphine to IS versus drug concentration (*C*) were found to be linear over the concentration range 10-200 ng mL^{-1} and 7.5-300 ng mL^{-1} using a weighted least squares method. The linear regression equations of the calibration curves of heroin and morphine were shown in Table 1.

Precision and Accuracy

The precision and accuracy of the method were examined by adding known amounts of heroin or morphine to blank human plasma. 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 results are summarized in Table 2. The intra-day and inter-day precisions were within 15%, which indicated that the method was reproducible. In statistical comparison ($P > 0.05$) with other methods in the literature[8,10,11,13] the proposed method has indicated high accuracy and precision.

Sensitivity

The sensitivity was evaluated by the limit of quantification (LOQ), the lowest concentration of the plasma spiked with heroin or morphine 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 heroin or morphine. Under the experimental conditions, the LOQ values were 10 ng mL^{-1} and 7.5 ng mL^{-1} heroin and morphin, respectively. Also, the LOD values were 5 ng mL^{-1} for heroin and 3 ng mL^{-1} for morphine, respectively.

Recovery

Extraction recovery was determined using the extraction and analysis of three control levels of analyte, at high, medium and low levels of the calibration range. Each control level was prepared

Table 3: Recovery of heroin and morphine from human plasma (n=6)

Sample	Concentration (ng mL^{-1})		%RSD	%RE
	Added	Found (Mean ± SD)		
Heroin	12.5	11.86 ± 0.236	94.9	1.99
	75	72.58 ± 1.362	100.7	1.88
	175	170.26 ± 2.046	97.3	1.2
Morphine	7.5	7.34 ± 0.286	97.9	3.89
	75	70.58 ± 1.532	94.1	2.17
	250	242.62 ±3.164	97	1.30

Table 4: Stability of heroin and morphine in human plasma (n=3)

Treatment	Recovery (Mean ± SD)					
	Heroin (ng mL^{-1})			Morphine (ng mL^{-1})		
	50	100	200	50	150	250
Three freeze-thaw cycles	93.56 ± 2.809	92.46 ± 2.974	94.15 ± 2.064	95.06 ± 2.644	93.48 ± 2.929	91.35 ± 2.926
Stored at RT for 8h[a]	92.59 ± 2.452	92.27 ± 3.123	94.53 ± 3.175	92.24 ± 3.224	96.53 ± 2.912	93.03 ± 3.263
Stored at -20 ^{0}C for 1 week	85.27 ± 3.562	87.47 ± 2.536	89.64 ± 3.775	87.46 ± 2.926	88.34 ± 4.921	86.51 ± 5.124

[a]RT, room temperature

with an "*n*" of 6. It was determined that liquid-phase extraction process was necessary at the sample preparation procedure. Several solvents (butyl chloride, ethylacetate, hexane, dichloromethane, acetonitrile and butanol) were tested for the extraction. Finally, ethylacetate proved to be the most efficient in extracting heroin and morphin in human plasma.

The recovery was determined by comparing peak area of heroin or morphin after extraction to that before extraction at concentrations of 12.5, 75, 175 and 7.5, 75, 250 ng mL^{-1}. The mean extraction recoveries of heroin and morphin in human plasma were 97.63% and 96.33%, respectively. The mean relative recovery for IS at 500 ng mL^{-1} was 95.24% (n = 6). Recovery data are shown in Table 3. Heroin or morfine were extracted from plasma with a solid phase extraction procedure by Rook *et al.,*[13] and Lee *et al.,*[11]. These methods are also the most comprehensive method which can extract heroin or morfine in a single extraction procedure. The mean recovery is better for plasma than those of the studies reported by Rook *et al.,*[13] and Lee *et al.,*[11].

Stability

The stability of heroin and morphin in human plasma was assessed by analyzing low, medium 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, medium and high concentration samples at room temperature for 8 h. Freze-thaw stability (-20 ^{0}C in plasma) was checked through three cycles. Samples were stored at -20 ^{0}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 freze-thaw cycles. The long-term stability was assessed after storage at -20 ^{0}C for 1 week. The results of the stability studies were given in Table 4 and no significant degradation of heroin and morphin was observed under the tested conditions.

Today, GC-MS is a powerful technique for highly specific and quantitative measurements of low levels of analytes in biological samples. As compared to HPLC, high-resolution capillary GC has

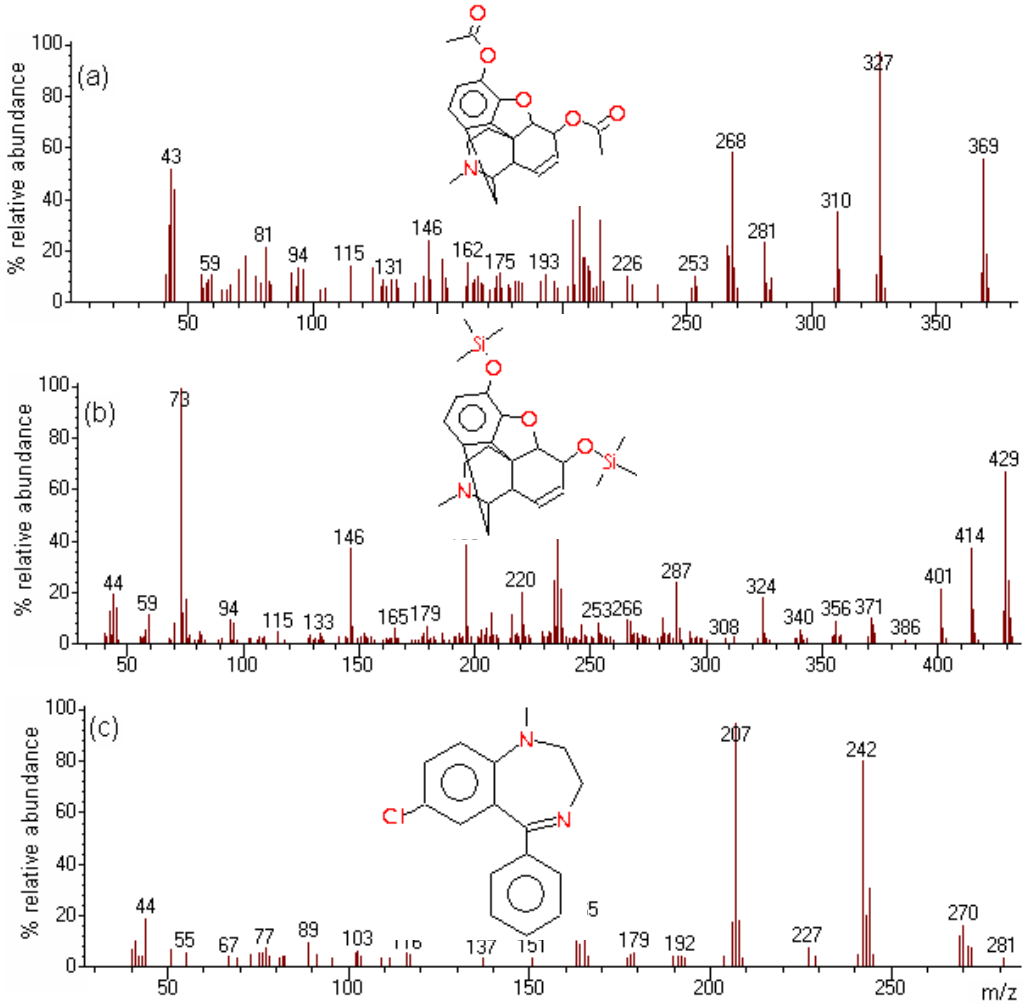

Fig. 1: Structural formula and MS spectra of heroin (a), morphine-di-TMS (b) and medazepam (IS) (c)

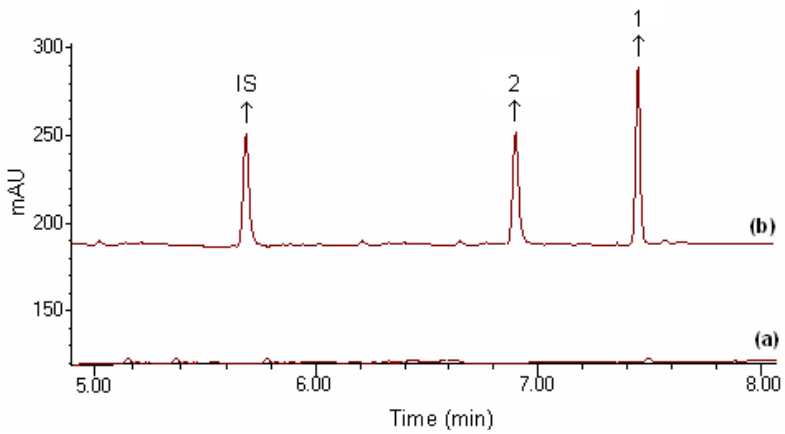

Fig. 2: Representative chromatograms of (a) drug-free human plasma, (b) the human plasma spiked with heroin (200 ng/mL), morphine-di-TMS (150 ng/mL) and IS (500 ng/mL), (1) Heroin (2) Morphine

been less frequently used[15,16]. However, it has inherently high resolving power and high sensitivity with excellent precision and accuracy allowed simultaneous detection of heroin and morphine. Also, the detection limits were lowered to ng levels by GC combined with MS.

Rook *et al.,*[13] have reported LC method with mass detection for the analysis of heroin and morphine in human plasma. The method was validated over a concentration range of 5-500 ng mL^{-1} for all analytes. The total recovery of heroin varied between 86 and 96% and of the heroin metabolites between 76 and 101%. Intra-assay and inter-assay accuracy and precision of all analytes were always within the designated limits (≤20% for limit of quantification (LOQ) and ≤15% for other samples). Detection using LC-MS would be a more sensitive approach but is costly and not yet available for every laboratory.

CONCLUSION

We successfully developed a simple GC-MS method for simultaneously assaying heroin and its metabolite morphin in human plasma. The assay has been validated with respect to accuracy, precision, linearity and limit of quantification, recovery and stability. Also, the extraction procedure in this study was simple. No significant interferences and matrix effect caused by endogenous compounds were observed. This is important for forensic purposes. Because this procedure allows to detect heroin and its metabolite morphin after ingesting an overdose of the drug.

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REFERENCES

1. Salmon, A.Y., Goren, Z., Avissar, Y., Soreq, H., *Clin Exp Pharmacol Physiol* **26:** 596 (1999).
2. Inturrisi, C.E., Schultz, M., Shin, S., Umans, J.G., Angel, L., Simon, E.J., *Life Sci* **33:** 773 (1983).
3. Hartley, R., Green, M., Quinn, M., Levene, M.I., *Biomed Chromatogr* **7:** 34 (1993).
4. Spector, S., *Meth Enzymol* **84:** 551 (1982).
5. Chapman, D.J., Joel, S.P., Aherne, G.W., *J Pharm Biomed* 1**2:** 353 (1994).
6. Vanderslooten, E.P.J., Vanderhelm, H.J., *Clin Chem* **22:** 1110 (1976).
7. Aoki, K., Shikama, Y., Kokado, A., Yoshida, T., Kuroiwa, Y., *Forensic Sci Int* **81:** 125 (1996).
8. Mabuchi, M., Takatsuka, S., Matsuoka, M., Tagawa, K., *J Pharm Biomed Anal* **35:** 563 (2004).
9. Whittington, D., Kharasch, E.D., *J Chromatogr B* **796:** 95 (2003).
10. Edwards, S.R., Smith, M.T., *J Chromatogr B* **814:** 241 (2005).
11. Lee, M.R., Yu, S.C., Hwang, B.H., Chen, C.Y., *Anal Chem Acta* **559:** 25 (2006).
12. Krogh, M., Christophersen, A.S., Rasmussen, K.E., *J Chromatogr Biomed* **621:** 41 (1993).
13. Rook, E.J., Hillebrand, M.J.X., Rosing, H., Van Ree, J.M., Beijnen, J.H., *J Chromatogr B* **824:** 213 (2005).
14. Proceedings of the International Conference on Harmonization (ICH), Commission of the European Communities, (1996).
15. Yilmaz, B., Arslan, S., Akba, V., *Talanta* **80:** 346 (2009).
16. Yilmaz, B., Arslan, S., *Chromatographia* **70:** 1399 (2009).