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Short communication

Simple and sensitive determination of free and total morphine in human liver and kidney using gas chromatography–mass spectrometry

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Abstracts

We developed a reliable, simple and sensitive method to determine free and total morphine in human liver and kidney, using gas chromatography–mass spectrometry (GC–MS). Free morphine or total morphine obtained by acid hydrolysis from 0.2 g tissue sample was extracted using an Extrelut® NT column with an internal standard, dihydrocodeine, followed by trimethylsilylation. The derivatized extract was submitted to GC–MS analysis of EI-SIM mode. The calibration curves of morphine in both liver and kidney samples were linear in the concentration range from 0.005 to 5 μg/g. The lower limits of detection of morphine were 0.005 μg/g. This method proved successful when we determined free and total morphine in liver and kidney obtained from an autopsied man who was mis-ingested morphine compound in the hospital, which resulted in the cause of death being morphine intoxication.
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1. Introduction

Analysis of morphine in human solid tissues, such as liver and kidney is particularly important when biological fluids are not available due to putrefaction of the body in suspected cases of morphine or heroin poisoning. When time has passed after ingestion, glucuronide and sulfate conjugation of morphine can be significant; hence, it is difficult to estimate the amount of drug ingested based on only the concentrations of unconjugated morphine in human fluids [1]. In such cases, total morphine concentrations obtained by acid hydrolysis have been used for forensic toxicological examinations. Therefore, it is important to develop a simple and reliable method to determine free and total morphine in human solid tissues.

Although there are many methods for the analysis of free morphine and total morphine in biological fluids using gas chromatography–mass spectrometry (GC–MS) [2–4], liquid chromatography [5,6] and liquid chromatography–mass spectrometry [7], only a few methods are available for use with solid tissues. Felby et al. [8] and Steentoft et al. [9] determined total morphine concentrations in the liver of fatal poisoning cases using a three step liquid–liquid extraction procedure and GC, where 20 g of tissues were required for analysis. Spiehler and Brown [10] also used a solvent extraction procedure for the analysis of total morphine in the liver and analyzed by GC–MS. Recently, a simpler method was developed using a mixed-mode solid phase column and was applied to the analysis of liver samples as well as biological fluids [11,12]. To our knowledge, studies such as selectivity, linearity, detection limit, accuracy and recovery focused on solid tissues are quite limited. Predmore et al. studied the recovery of morphine from liver by hydrolysis and solvent extraction using radioactively tagged morphine [13]. Cingolani et al. [12] presented validation data of their method developed for the analysis of total morphine in fixed tissues using 5 g of liver sample.

Such being the case, we developed a more simple and well working GC–MS method to determine free and total morphine in human liver and kidney.

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2. Experimental

2.1. Reagents

Morphine hydrochloride was purchased from Sankyo (Tokyo, Japan) and dihydrocodeine phosphate was purchased from Takeda Chemical Industries (Osaka, Japan). BSTFA with 1% TMCS and pyridine (silylation grade) were purchased from Pierce (Milwaukee, WI, USA). Extrelut® NT Refill Pack was purchased from Merck (Darmstadt, Germany). The powder (3.0 g each) was packed in a 1.5 cm diameter glass column, and each sample was directly applied to the column without steps of conditioning. Dichloromethane and isopropyl alcohol were distilled prior to use. Other chemicals used were of analytical reagent grade.

2.2. Biological samples

Human tissue samples obtained at the time of autopsy were stored at −20 °C until analysis and drug-free human tissues were used as control samples.

2.3. Standard solutions

Morphine hydrochloride (5.6 mg) was dissolved in methanol and the volume was adjusted to 5 ml, to obtain a concentration of 1 μg/μl as a free base. This solution was further diluted in methanol to 0.1, 0.01 and 0.001 μg/μl, respectively. Dihydrocodeine phosphate (6.6 mg) was dissolved in methanol, in the same manner.

2.4. Extraction and derivatization procedure

Liver or kidney sample (0.2 g each) was mixed with 1.5 ml of 0.01 M hydrochloric acid and 2 μl of IS solution (0.2 μg dihydrocodeine) in a round bottom centrifuge tube (12 ml, Nalge company, New York U.S.A.), then this mixture was homogenized by POLYTRON® PT3100 (KINEMATICA AG, Littau-Luzern, Switzerland) and centrifuged at 850 × *g* for 15 min. After the supernatant had been transferred to another centrifuge tube (12 ml), the pH was adjusted to 9.5 by adding 1 ml of 1 M sodium carbonate–sodium hydrogen carbonate buffer (pH 9.5). The mixture was vortex-mixed for 10 s, then applied to an Extrelut® NT column. After standing for 20 min, morphine and IS were eluted with 12 ml of the mixture of dichloromethane–isopropyl alcohol (85:15). The eluate was evaporated to dryness under a stream of nitrogen. The residue was dissolved in 50 μl of pyridine, and 50 μl of BSTFA with 1% TMCS was added to the solution for trimethylsilylation. The mixture was kept at 90 °C for 30 min, cooled down to a room temperature, and a 2-μl aliquot of the solution was injected onto a GC–MS apparatus.

For the analysis of total morphine, acid hydrolysis was carried out as reported [14]. A half millilitre of 37% hydrochloric acid was added to the supernatant obtained as described above, and heated at 90 °C for 30 min. The mixture was cooled down to a room temperature, and 0.75 ml of 10 M sodium hydroxide and 1 ml of 2.3 M ammonium sulfate was added to the mixture in order to adjust the pH to about 9. Then the mixture was applied to an Extrelut® NT column, derivatized and injected onto a GC–MS apparatus with the same manner used for morphine analysis.

2.5. GC–MS conditions

The apparatus used was an Agilent 5973 GC–MS system. An HP-1ms fused-silica capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness) coated with 100% dimethylpolysiloxane stationary phase was used. Splitless injection mode was selected with a valve off time of 2 min. The GC–MS conditions were as follows: the initial temperature 100 °C was held for 2 min, the temperature was programmed to 300 °C at a rate of 20 °C/min; this temperature being maintained for 3 min. Injection port and transfer line temperatures were 250 and 280 °C, respectively. Helium with a flow rate of 1 ml/min was used as the carrier gas.

The selected ion monitoring (SIM) mode was used. The ions *m*/*z* 429 and 414 were used as qualifier ions for morphine, and *m*/*z* 373 and 315 for IS, respectively. As quantifier ions *m*/*z* 429 and *m*/*z* 373 were used for morphine and IS, respectively.

2.6. Preparation of calibration curves

Liver and kidney samples were prepared to contain morphine at concentrations of 0.005, 0.025, 0.05, 0.25, 0.5, 2.5 and 5 μg/g, each containing 1 μg/g IS. These samples were extracted in the same manners as described above. The calibration curve was obtained by plotting the peak-area ratio of morphine derivative to IS derivative versus the amount of morphine.

3. Results and discussion

3.1. Extraction procedure

Since a one-step extraction procedure for morphine with dichloromethane gave many interfering peaks on the chromatogram in the analysis of liver and kidney samples, we first applied a three step liquid–liquid extraction procedure used for the analysis of common basic drugs [15]. However, constant recovery of morphine was not obtained probably due to amphoteric nature of this drug. Comparative studies of different extractive procedures to quantify morphine in various biological fluids have been published [3,16,17]. Soares et al. [16] examined three extraction procedures: Sep-Pak® C_{18} cartridge, Extrelut® column and liquid–liquid extraction with further purification through Extrelut® column, and found that the last procedure gave the cleanest extracts for the analysis of urine by HPLC. Reversed-phase (C_{18}) extraction gave better sample purification compared with liquid–liquid or Extrelut® extraction in the analysis of human vitreous humor by GC–MS [17]. Since no studies focusing on the extraction procedure of morphine in solid tissues were found, we chose an Extrelut® NT column based on our experience in the analyses of methomyl [18] and bromvalerylurea [19] using this column. Extrelut® column, a well known sample preparation device that can be used in

place of liquid–liquid extraction procedure, could absorb colored interfering compounds in solid tissues completely and clean extracts were obtained. As the particles of Extrelut® which is made of diatomaceous earth are much bigger than those of silica or polymer-based columns, and packed more loosely, the problem of column plugging often occurred in the analysis of solid tissues with use of silica or polymer-based columns was also overcome. Several eluting solvents, ethyl acetate, dichloromethane and mixtures of dichloromethane–isopropyl alcohol at different ratios were examined, and the mixture of dichloromethane–isopropyl alcohol at the ratio of 85:15 was found to give best recovery of morphine. As for IS, we used dihydrocodeine. Although the deuterium-labeled morphine is the most common IS used for analysis of morphine by GC–MS (2–3), dihydrocodeine is useful as an IS in cases where deuterium-labeled morphine is not commercially available.

For the analysis of total morphine, pH adjustment to about 9 after acid hydrolysis was essential as the recovery of morphine significantly decreased at higher pH. Adjustment of pH was quickly carried out by adding strong alkali solution and ammonium sulfate as reported by Maurer et al. [14], and time was saved.

3.2. Determination of morphine and total morphine in liver and kidney by GC–MS

Electron impact (EI) mass spectra of TMS derivatives of morphine and IS are shown in Fig. 1. Major fragment ions were observed at *m/z* 429, 414, 401, 324, 287 and 236 for morphine and *m/z* 373, 358, 315, 282 and 236 for IS. Each ion was examined and ions of *m/z* 429 and 414 for morphine and *m/z* 373 and 315 for IS were selected as qualifier ions, and *m/z* 429 and 373 were used as quantifier ions for morphine and IS, respectively. Figs. 2 and 3 shows SIM chromatograms of derivatized extracts from liver and kidney spiked with 0.05 μg/g of morphine and 1 μg/g of IS and from blank liver and kidney containing only IS. Each peak was clearly separated on the chromatograms with retention times of 12.18 and 11.64 min, respectively. There were

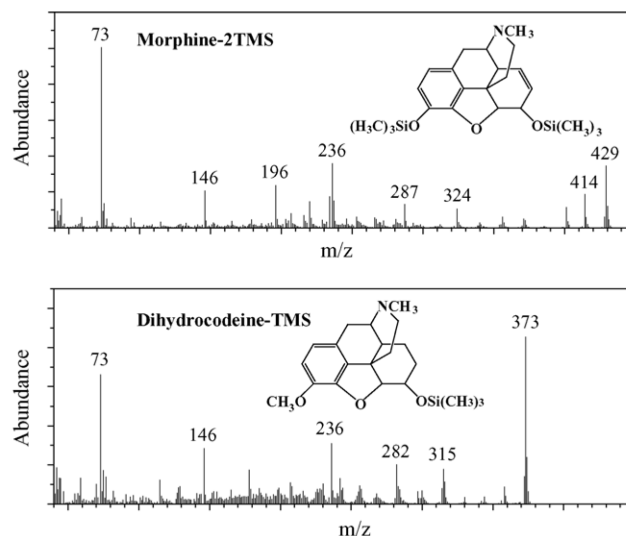

Fig. 1. EI mass spectra of TMS derivatives of morphine and IS.

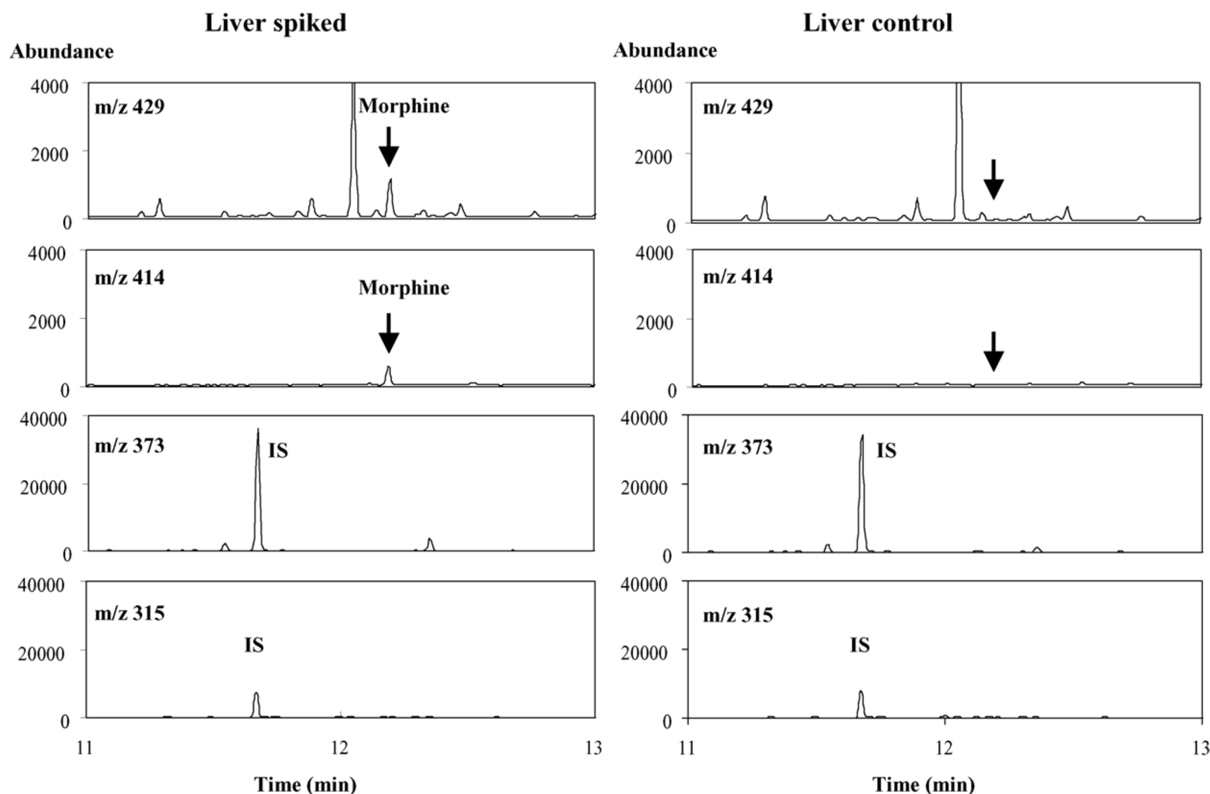

Fig. 2. SIM chromatograms of derivatized extracts from a human liver spiked with 0.05 μg/g of morphine and from a control liver.

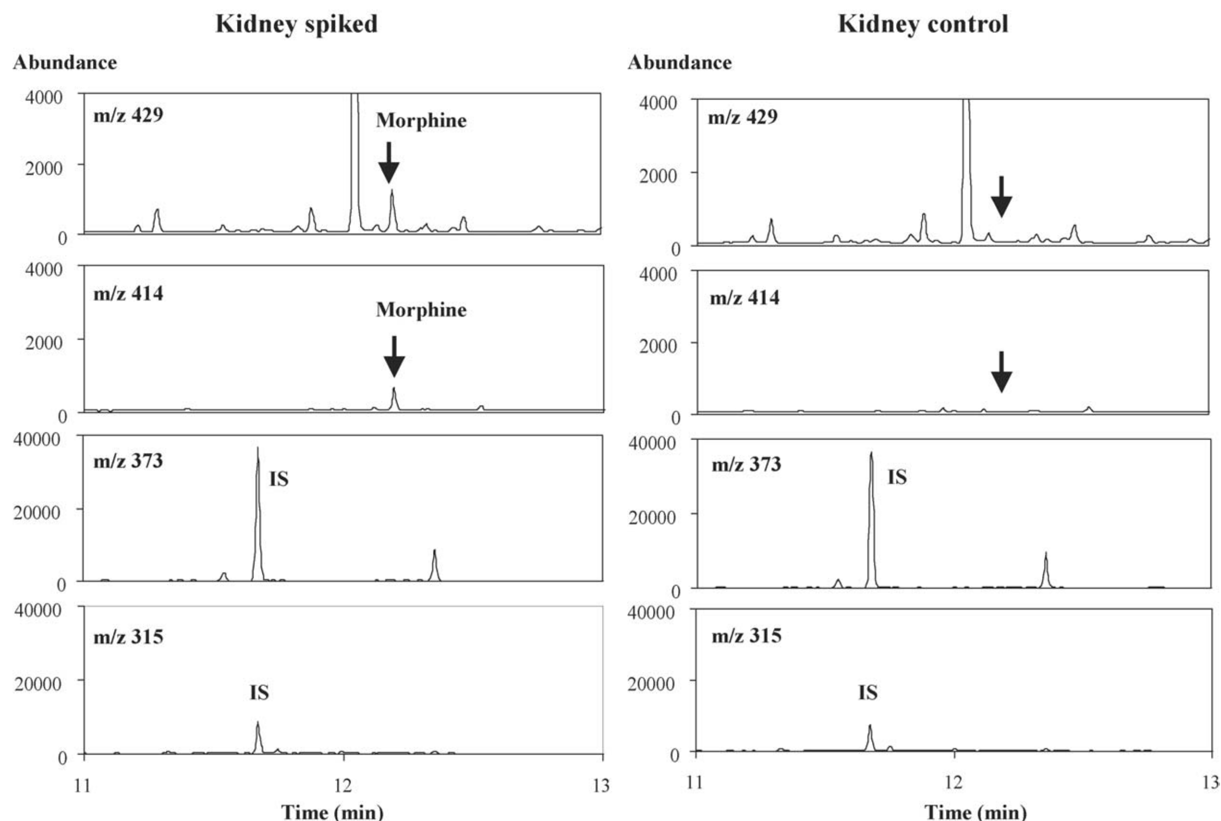

Fig. 3. SIM chromatograms of derivatized extracts from a human kidney spiked with 0.05 μg/g of morphine and from a control kidney.

no interfering peaks on the chromatograms of blank samples. In the samples for the analysis of total morphine, the similar but cleaner chromatograms were obtained. This may be because that the tissue materials were completely decomposed by strong acid and less organic compounds were extracted by the solvent. The calibration curves were linear in the concentration range from 0.005 to 5 μg/g in liver and kidney samples with correlation coefficients exceeded 0.99. Linear regression analyses gave the equations, $y = 0.6617x - 0.015$ (liver) and $y = 0.6239x - 0.0146$ (kidney) for free morphine, and $y = 0.6923x - 0.023$ (liver) and $y = 0.6592x - 0.0138$ (kidney) for total morphine with the correlation coefficients exceeded 0.99 (x = the analyte concentration (μg/g), y = peak-area ratio). The lower limits of detection for morphine, at a signal-to-noise ratio of 3, were 5 ng/g in both liver and kidney samples when 0.2 g of sample was submitted to analysis. The absolute recoveries of morphine in liver and kidney at two different concentrations, 0.05 and 0.5 μg/g, were determined triplicate by comparing the peak-areas of derivatives of morphine in samples with those in standard solutions. The average recoveries for free morphine in liver samples at above two concentrations were 60.4 and 53.6%, respectively. In kidney samples, the recoveries were 69.6 and 52.4%, respectively. The average recoveries for total morphine at 0.05 and 0.5 μg/g were 90.3 and 77.6% in the liver and 83.7 and 99.1% in the kidney, respectively. Within-day precision of this method in liver and kidney at concentrations of 0.05 and 0.5 μg/g was examined. The relative standard deviations ($n = 5$) were 3.7 and 3.0% for liver and 4.3 and 5.3% for kidney, respectively (Table 1). In total morphine, the relative standard deviations ($n = 5$) were obtained by the analyses of morphine-intoxicated tissues in order to know the variation including acid hydrolysis step. The obtained deviations were 8.6% for liver and 7.2% for kidney.

Table 1
Accuracy and precision of free morphine determination in human liver and kidney

Sample	Added (μg/g)	n	Detected (μg/g)		R.S.D. (%)	Accuracy (%)
			Mean	S.D.		
Liver	0.05	5	0.054	0.002	3.7	108.0
	0.50	5	0.508	0.015	3.0	101.6
Kidney	0.05	5	0.047	0.002	4.3	94.0
	0.50	5	0.492	0.026	5.3	98.4

Therefore, this method is reliable and can be used for diagnosis of morphine and/or heroin poisoning. Furthermore, as this method requires only 0.2 g of tissues, it is also applicable for pharmacokinetic studies using small animals.

4. Practical application

Our method was applied for quantitative determinations of free and total morphine in liver and kidney of a patient who had mistakenly been given 150 mg of morphine prepared for another

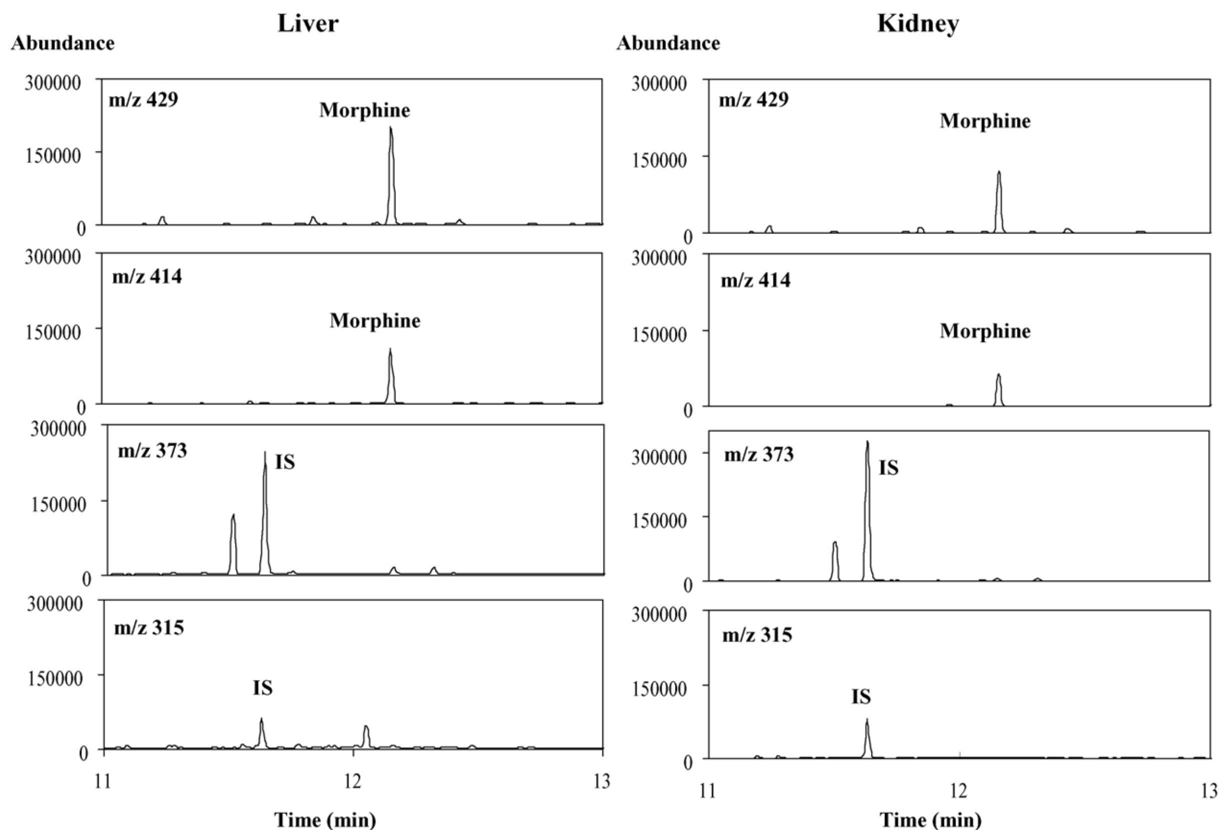

Fig. 4. SIM chromatograms of derivatized extracts from liver and kidney of an autopsied man who died after mistakenly being given morphine.

terminally ill cancer patient. His condition suddenly changed a half day after being given this morphine and he died 3 days later. Since 3 days passed after this drug administration, liver and kidney samples were submitted to analysis with other biological fluids in order to accurately diagnose the cause of death. Free morphine and total morphine were clearly detected in both samples with no interfering peaks. Fig. 4 shows SIM chromatograms of derivatized extracts after acid hydrolysis of the liver and kidney samples collected from the patient. The concentrations of free morphine and total morphine were 0.157 and 0.535 μg/g in the liver, and 0.087 and 0.478 μg/g in the kidney, respectively.

5. Conclusions

We developed a simple and reliable GC–MS method to determine free and total morphine in human liver and kidney. This is well validated procedure which can be applied to forensic toxicological cases as well as pharmacokinetic studies in small animals.

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