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High-performance liquid chromatography-mass spectrometry-mass spectrometry analysis of morphine and morphine metabolites and its application to a pharmacokinetic study in male Sprague–Dawley rats[1]

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Abstract

A high-performance liquid chromatography tandem mass spectrometry-mass spectrometry (LC-MS-MS) assay was developed for the analyses of morphine, morphine glucuronides and normorphine in plasma samples from rats. The analytes were extracted by using C_2 solid-phase extraction cartridges. The extraction recoveries were 100% for morphine, 84% for morphine-3-glucuronide, 64% for morphine-6-glucuronide and 88% for normorphine. Both intra- and inter-assay variabilities were below 11%. Using a plasma sample size of 100 μl, the limits of detection were 13 nmol l^{-1} (3.8 ng ml^{-1}) for morphine, 12 nmol l^{-1} (5.5 ng ml^{-1}) for morphine-3-glucuronide, 26 nmol l^{-1} (12 ng ml^{-1}) for morphine-6-glucuronide and 18 nmol l^{-1} (5.0 ng ml^{-1}) for normorphine, at a signal-to-noise ratio of 3. The present assay was applied to a pharmacokinetic study in rats after intraperitoneal administration of morphine.

Keywords: Morphine; Morphine-3-glucuronide; Morphine-6-glucuronide; Normorphine; LC-MS-MS; Pharmacokinetics

1. Introduction

Morphine is the most frequently used opioid analgesic in the pharmacological intervention of moderate to severe cancer pain. After the administration, morphine undergoes extensive metabolism which primarily occurs in liver. Glucuronidation is the main metabolic pathway which produces morphine-3-glucuronide and morphine-6-glucuronide as the major metabolites. The urinary recoveries of morphine-3-glucuronide and morphine-6-glucuronide account for 55% and 10% of the given dose, respectively [1]. Morphine-6-glucuronide exhibits similar affinity for μ-opioid

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receptors to that of morphine [2,3]. It is a more potent analgesic than morphine in rodents [4,5] and an effective and well-tolerated analgesic when tested in healthy volunteers [6] as well as in cancer patients [7–9]. In contrast, morphine-3-glucuronide has very low or no affinity for opioid receptors [10] and is devoid of analgesic effect by itself [11,12]. It may, however, functionally antagonize morphine or morphine-6-glucuronide induced antinociception [13,14] and play a role in the development of tolerance to the antinociceptive effects of morphine [12,15]. The *N*-demethylated metabolite, normorphine, is a minor metabolite of morphine which is equipotent with morphine in spinal antinociception in rats [16].

Various analytical assays have been developed for the purposes of pharmacokinetic studies and forensic analyses of morphine. By and large, radioimmunoassays and reverse-phase high performance liquid chromatography (HPLC) are the most widely used methods. Although radioimmunoassays [17–19] have the advantages of high sensitivity, high throughput of the samples and simplicity, the cross-reactivity among morphine, morphine metabolites and their analogs is frequently a concern for assay validity, especially in cases such as pharmacokinetic studies and forensic analyses. HPLC allows simultaneous separation of morphine and its metabolites which can be detected by ultraviolet (UV) spectrophotometric detection [20–23], electrochemical detection (ECD) [24,25], fluorescence detection [26–28], combined ECD-UV detection [29] or combined ECD-fluorescence detection [30–32]. Solid-phase extraction was used in all cases to extract morphine and its metabolites at the same time.

Recently, HPLC tandem mass spectrometric assays (LC-MS) have been developed for the analyses of morphine and morphine glucuronide metabolites in urine [33], and serum samples [34,35]. The samples were processed by using either C_{18} [33,35] or C_2 [34] solid-phase extraction cartridges and morphine and its glucuronide metabolites were determined by selected ion monitoring (SIM). Using a sample size of 1 ml, the limits of detection were as low as 0.84, 5.0 and 2.0 ng ml^{-1} for morphine, morphine-3-glucuronide and morphine-6-glucuronide, respectively [35].

Although SIM was used as a means of detection in the above LC-MS analyses, it is still prone to various interfering substances present in biological samples, which is especially true in cancer patients who are usually on multi-drug therapies. MS-MS analysis involves the simultaneous monitoring of both parent ion(s) and daughter ion(s) in the two sequentially coupled mass spectrometers, respectively. The combined selectivities of effective separation by HPLC and ion monitoring of both parent and daughter ions by MS-MS would provide unequivocal proof to the assay results, an aspect which is extremely important for forensic and drug doping analyses. To our knowledge, LC-MS-MS assay for the analysis of morphine and its metabolites has not been reported. We thus report here a highly selective and sensitive LC-MS-MS assay developed for the simultaneous determination of morphine, morphine-3-glucuronide, morphine-6-glucuronide and normorphine and its application in a pharmacokinetic study in rats.

2. Experimental

2.1. Chemicals

Morphine-3-glucuronide crystalline and morphine-6-glucuronide dihydrate were obtained from Sigma (St. Louis, MO, USA). Morphine sulfate pentahydrate and formic acid (98%) were obtained from BDH Inc. (Toronto, Ont., Canada). Normorphine hydrochloride and nalorphine hydrochloride were obtained from the Health Protection Branch of Canada (Ottawa, Ont.). Ammonium bicarbonate and ammonium hydroxide (30%) were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Purified water was prepared using a Milli-Q water purification system (Mississauga, Ont.). All other solvents were of HPLC grade.

2.2. Stock solutions

A stock solution equivalent to 224 nmol (63.8 μg) of morphine base, 149 nmol (68.6 μg) of morphine-3-glucuronide and 159 nmol (73.3 μg)

of morphine-6-glucuronide was prepared in 50 ml of purified water. A stock solution containing 1.9 μmol (507 μg) of normorphine base was prepared in 50 ml of purified water, which was further diluted to 1.8 nmol ml^{-1} (500 ng ml^{-1}) with purified water. The nalorphine internal standard solution was prepared by dissolving 1.5 μmol (457 μg) of nalorphine hydrochloride in 100 ml of purified water.

Ammonium bicarbonate buffer (0.01 M, pH 9.3) was freshly prepared by dissolving 0.4 g of ammonium bicarbonate in 470 ml of purified water, adjusting pH to 9.3 with ammonium hydroxide, and diluting with purified water to 500 ml.

2.3. LC-MS-MS conditions

The LC system consisted of a HP series II 1090 Liquid Chromatograph (Hewlett Packard, Avondale, PA, USA), a Zorbax SB-phenyl column (5 μm, 250 × 4.6 mm I.D.) (Rockland Technologies Inc., Newport, DE, USA), a guard column equipped with a Brownlee phenyl cartridge (7 μm, 15 × 3.2 mm I.D.) (Santa Clara, CA, USA). The mobile phase consisted of 0.1% (v/v) formic acid in 18% of methanol (methanol-water, 18:82, v/v) which was delivered at a flow rate of 0.7 ml min^{-1}. The LC effluent was split and 10% of the effluent was introduced into the MS.

The MS-MS system was a VG Quattro triple quadrupole mass spectrometer equipped with an electrospray interface (Fisons, Altrincham, UK). The electrospray source was operated with a capillary voltage of 3.4 kV, a cone voltage of 40 V and a source temperature of 130°C. Nitrogen was used as both a nebuliser gas and a drying gas with flow rates of 15 and 250 l h^{-1}, respectively. The MS-MS analyses was performed in the Multiple Reaction Monitoring (MRM) mode. The first MS (MS1) was static at pre-selected protonated parent ion masses. The selected parent ions were introduced into the collision cell and the fragmentation occurred at a collision energy of -80 eV. Argon was used as a collision gas at a pressure around 4×10^{-4} mbar. The second MS (MS2) was static at pre-determined daughter ion masses produced from the fragmentation in the collision cell. The low mass (LM) and high mass (HM) resolution of both MS were set at 5.0. A dwell time of 0.4 s was used for the scanning. The MS/MS parameters were optimized by injecting a standard solution containing morphine, morphine-3-glucuronide, morphine-6-glucuronide and normorphine under various conditions. Mass calibration was performed by using sodium iodine and caesium iodine.

The operation of the LC-MS-MS system was controlled by computer software MassLynx 2.1 (Fisons PLC).

2.4. Procedures of solid-phase extraction

The samples were extracted according to Pacifici et al. [34] with modification. To 100 μl of plasma in a 0.6-ml polypropylene microcentrifuge tube (Fisher, Pittsburgh, PA, USA) were added 5.0 μl of the nalorphine internal standard solution and 0.1 ml of the ammonium bicarbonate buffer and the contents mixed manually. The mixture was loaded onto a C_2 solid-phase extraction cartridge (1 ml/100 mg, Bond Elut, Varian, Harbor City, CA) which was pre-conditioned with 2 ml of methanol, 1 ml of water and 1 ml of the ammonium bicarbonate buffer. The C_2 cartridge was connected to a Baker spe-10 vacuum manifold (J.T. Baker Inc., Toronto). The cartridge was vacuum-dried for 30 s, washed with 1 ml of the ammonium bicarbonate buffer, and again vacuum-dried for 2 min. The analytes were eluted with 1 ml of methanol which was subsequently evaporated to dryness under a gentle stream of nitrogen. The residues were reconstituted in 100 μl of the HPLC mobile phase by vortex-mixing at high speed for 1 min followed by centrifugation at $2800 \times g$ for 5 min. The supernatant was transferred into a 0.35-ml glass insert (Kimble Glass Inc., Vineland, NJ) and an aliquot of 10 μl was used for LC-MS-MS analyses.

2.5. Calibration curves

The calibration curves for morphine, morphine-3-glucuronide and morphine-6-glucuronide were determined in a series of blank 100-μl plasma samples to which were added 5.0 μl of the nalor-

phine internal standard solution and 0, 0.30, 0.40, 1.0, 2.0, 5.0, 20, 80 and 160 μl of the stock solution containing the above three analytes. The samples were extracted as described in the Section 2.4. The calibration curve for normorphine was determined in another series of blank plasma samples in a similar manner, except the final concentrations of normorphine in the samples were 0, 18, 37, 74, 184, 369, and 738 nmol l^{-1} (0, 5.0, 10, 20, 50, 100 and 200 ng ml^{-1}), respectively. The calibration curves were constructed by plotting the peak area ratios of the analyte to the internal standard against the concentrations of the analyte in the samples.

2.6. Assay precision

The intra-assay variabilities of morphine, morphine-3-glucuronide and morphine-6-glucuronide were determined by the analyses of two sets of six 100-μl plasma samples to which 2.0 and 20 μl of the stock solution containing the above analytes were added, respectively. After the addition of 5.0 μl of the internal standard solution, the samples were extracted and analyzed as described on the same day. The intra-assay variability of normorphine was determined in another two sets of six 100-μl plasma samples in a similar manner, except the final concentrations of normorphine in the two sets of samples were 74 and 369 nmol l^{-1} (20 and 100 ng ml^{-1}), respectively.

The inter-assay variabilities of morphine, morphine-3-glucuronide and morphine-6-glucuronide were determined by the analyses of two sets of five 100-μl plasma samples to which 2.0 and 20 μl of the stock solution containing the above analytes were added, respectively. One sample at each concentration was extracted and analyzed every day over a consecutive 5-day period.

2.7. Extraction recoveries

The extraction recoveries of morphine, morphine-3-glucuronide and morphine-6-glucuronide were determined by the analyses of two sets of six 100-μl plasma samples to which 2.0 and 20 μl of the stock solution were added, respectively. The samples were extracted and analyzed as described before, except the internal standard was added during the final reconstitution. Standard solutions at the same concentrations along with the internal standard were prepared in parallel at the same time. The extraction recoveries were determined by comparison of the peak area ratios of morphine, morphine-3-glucuronide and morphine-6-glucuronide to the internal standard from extracted samples with those of morphine, morphine-3-glucuronide and morphine-6-glucuronide to the internal standard from the unextracted standard solutions. The extraction recovery of normorphine was determined in another two sets of six 100-μl plasma samples in a similar manner, except the final concentrations of normorphine in the two sets of samples were 74 and 369 nmol l^{-1} (20 and 100 ng ml^{-1}), respectively.

2.8. Pharmacokinetic study in rats

The experimental protocol was approved by the Committee on Animal Care of the University of British Columbia (UBC). Male Sprague Dawley rats ($n = 5$) were obtained from the Animal Care Center of UBC 6 days before the experiment. The rats were provided standard laboratory food and water ad libitum. The animal housing room was maintained at 24°C, with a 12-h light/dark cycle (lights on at 06:00 h). The rats weighed 268–292 g at the time of the experiment.

Morphine sulfate was dissolved in 0.9% sodium chloride solution at 14 μmol ml^{-1} (4 mg of morphine base ml^{-1}) and administered intraperitoneally (i.p.) at a dose of 14 μmol kg^{-1} to the rats. Blood samples of approximately 200 μl were obtained by transection of the tail, about 1–2 mm from the tip, at 5, 10, 15, 20, 30, 40, 80, 120 and 240 min after the administration, and collected by using 250-μl heparinized Natelson blood collecting tubes (Fisher Scientific). Plasma was separated, collected in 0.6 ml polypropylene microcentrifuge tubes, and kept at −20°C until analyses.

2.9. Calculation of pharmacokinetic parameters

The area under the curve (AUC) was calculated by using the trapezoidal method [36]. The maxi-

mum plasma concentrations (C_{max}) and the time for achieving the C_{max} (T_{max}) were observed values. The elimination rate constant of morphine (K_e) was calculated from the equation: $K_e = -2.3 \times a$, where a is the slope deduced by log-linear regression analysis of the last five data points using SigmaPlot (Jandel Scientific, San Rafael, CA). The elimination half-life of morphine ($t_{1/2}$) was thus calculated from: $t_{1/2} = 0.693/K_e$. Similarly, the slope of the elimination phase for morphine-3-glucuronide was calculated by log-linear regression analysis of the last four data points.

2.10. Statistical analyses

Statistical analyses were performed by the two-tailed t-test at the significance level of $P = 0.05$ using a statistical software package prepared by the Departments of Botany and Zoology, Faculty of Science, the University of British Columbia.

3. Results and discussion

To eliminate the contamination and plugging of the MS components by nonvolatile buffers in most MS interfaces, volatile buffers, such as ammonium acetate, ammonium formate and trifluoroacetic acid (TFA), are used in the preparation of mobile phases for LC separation. TFA and formic acid were tested as mobile phase modifiers in our study. TFA (0.05%, v/v) produced approximately one-tenth the sensitivity as obtained with formic acid (0.1%, v/v). Similar results have been reported by Tyrefors et al. [35]. Using a Zorbax phenyl column, 0.1% formic acid in 18% methanol provided good separation for the analytes and the internal standard (Fig. 1B). There were no interfering peaks detected in plasma samples obtained from control rats (Fig. 1A).

The solid-phase extraction using C_2 cartridges gave 100, 84, 64, and 88% of the recoveries for morphine, morphine-3-glucuronide, morphine-6-glucuronide, and normorphine, respectively (Table 1). The recoveries at the two concentrations for each analyte were not significantly different ($P > 0.05$).

The calibration curves were linear over the concentration ranges of 13–7165 nmol l^{-1} (3.8–2042 ng ml^{-1}) for morphine ($y = 1.6 \times 10^{-3}\ x - 8.6 \times 10^{-4}$, $r^2 = 0.9959$), 12–4761 nmol l^{-1} (5.5–2195 ng ml^{-1}) for morphine-3-glucuronide ($y = 1.8 \times 10^{-3}\ x - 6.5 \times 10^{-3}$, $r^2 = 0.9995$), 26–5089 nmol l^{-1} (12–2346 ng ml^{-1}) for morphine-6-glucuronide ($y = 3.9 \times 10^{-4}\ x - 1.3 \times 10^{-3}$, $r^2 = 0.9997$), and 18–738 nmol l^{-1} (5.0–200 ng ml^{-1}) for normorphine ($y = 1.0 \times 10^{-3}\ x\ 4.6 \times 10^{-3}$, $r^2 = 0.9991$). The limits of detection were 13 nmol l^{-1} (3.8 ng ml^{-1}) for morphine, 12 nmol l^{-1} (5.5 ng ml^{-1}) for morphine-3-glucuronide, 26 nmol l^{-1} (12 ng ml^{-1}) for morphine-6-glucuronide, and 18 nmol l^{-1} (5.0 ng ml^{-1}) for normorphine (signal-to-noise ratio of 3), by using a plasma sample size of 100 μl.

The intra-assay variabilities were below 10% for all of the four analytes, while the inter-assay variabilities were below 11% for morphine, morphine-3-glucuronide and morphine-6-glucuronide (Table 2) as determined over a 5-day period. Since the analysis of normorphine in the plasma samples from the pharmacokinetic study was completed on the same day, the inter-assay variability for normorphine was not determined.

The reason that the calibration curve and the assay precision for normorphine were done separately from those of morphine and morphine glucuronides was because normorphine was later detected after the completion of the analyses of morphine and morphine glucuronides in the rat's plasma samples from the pharmacokinetic study. The present assay can be used to analyze all four analytes at the same time, as shown in Fig. 1.

The present LC-MS-MS assay was applied to a pharmacokinetic study in male Sprague Dawley rats after i.p. administration of morphine at a dose of 14 μmol kg^{-1} (4 mg kg^{-1}). Fig. 1C shows mass chromatograms of a plasma sample from a rat collected at 10 min after the administration. Morphine, morphine-3-glucuronide and normorphine were detected from the sample whereas morphine-6-glucuronide was absent. As a matter of fact, morphine-6-glucuronide could not be detected in all of the samples which further confirms

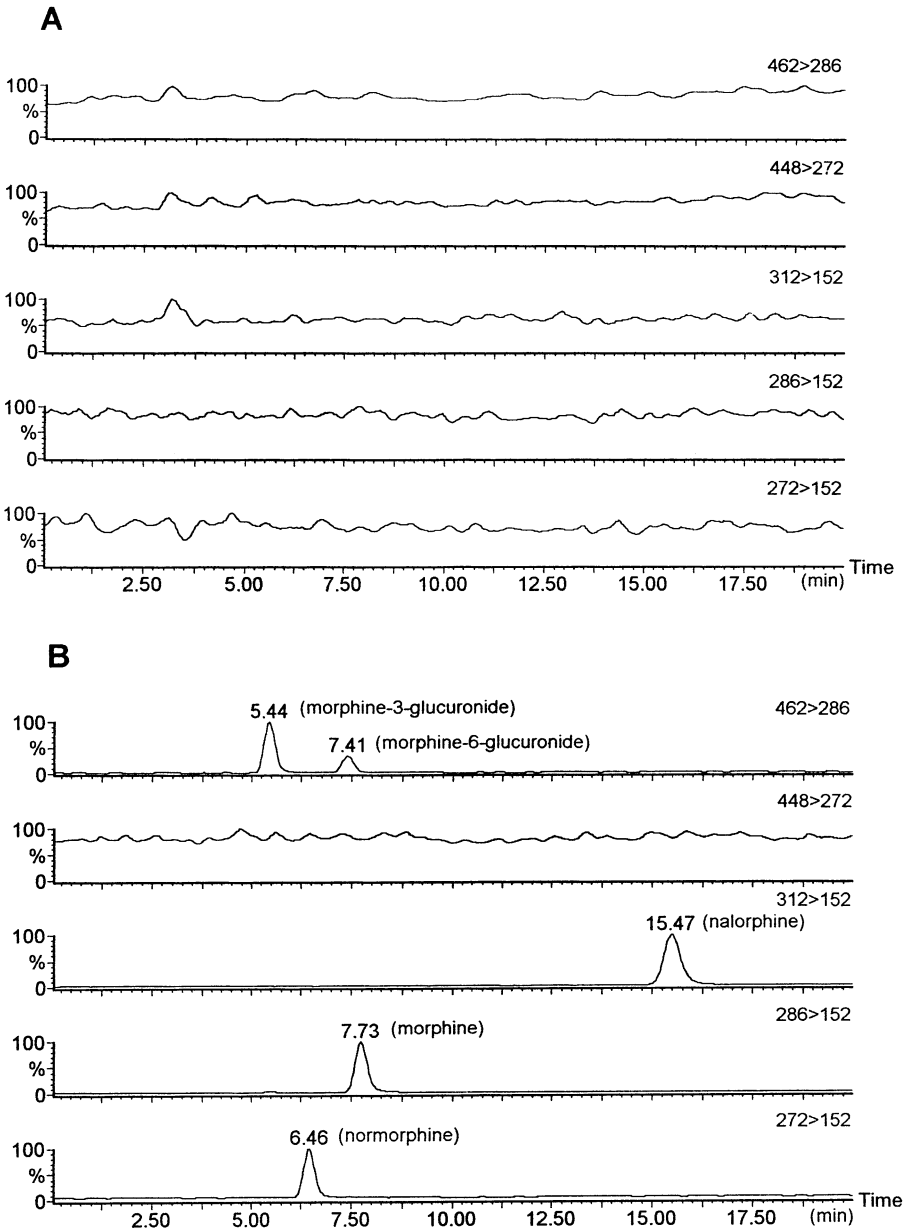

Fig. 1. LC-MS-MS chromatograms of (A) a blank rat's plasma sample, (B) a rat's plasma sample added the standards: morphine-3-glucuronide 297 nmol l^{-1} (137 ng ml^{-1}), morphine-6-glucuronide 319 nmol l^{-1} (147 ng ml^{-1}), nalorphine 570 nmol l^{-1} (200 ng ml^{-1}), morphine 449 nmol l^{-1} (128 ng ml^{-1}), and normorphine 369 nmol l^{-1} (100 ng ml^{-1}), and (C) a rat's plasma sample collected at 10 min after i.p. administration of morphine at a dose of 14 μmol kg^{-1} (4 mg kg^{-1}): morphine-3-glucuronide 3050 nmol l^{-1} (1406 ng ml^{-1}), normorphine glucuronide 54 nmol l^{-1} (24 ng ml^{-1}), nalorphine 570 nmol l^{-1} (200 ng ml^{-1}), morphine 1232 nmol l^{-1} (351 ng ml^{-1}), and normorphine 114 nmol l^{-1} (31 ng ml^{-1}). Plasma (100 μl) was used for all the analyses. The $462 > 286$, $448 > 272$, $312 > 152$, $286 > 152$ and $272 > 152$ channels were used to monitor morphine-3- and morphine-6-glucuronides, normorphine glucuronide, nalorphine internal standard, morphine and normorphine, respectively. The y-axis indicates relative intensities.

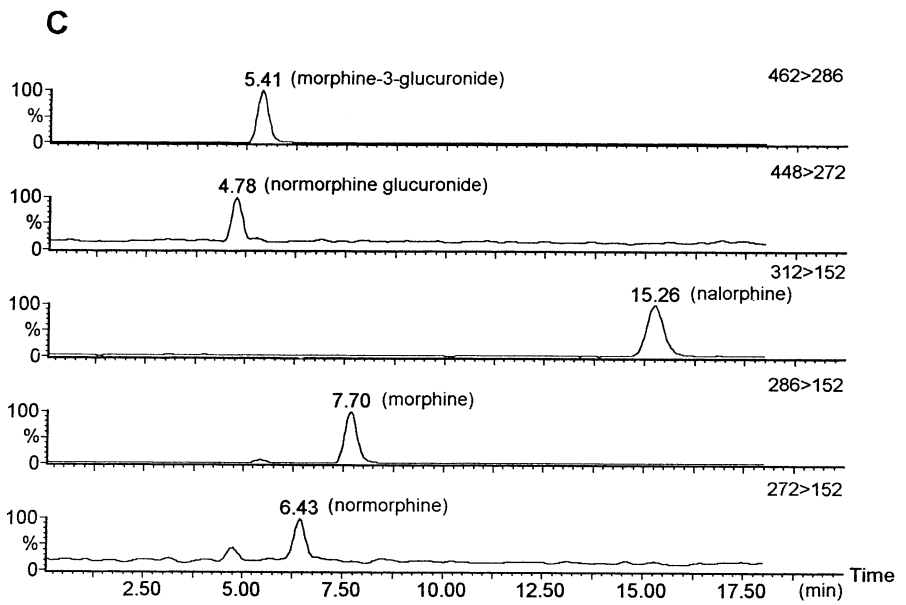

Fig. 1. (*Continued*)

the results reported by other investigators that morphine is not metabolized to morphine-6-glucuronide in rats [37,38].

The plasma concentration profiles of morphine and its metabolites are presented in Fig. 2. Following the administration, morphine was rapidly absorbed and reached T_{max} at 8.0 min (Table 3). The elimination half-life of 41 min is in accordance with the literature value [39]. The $AUC_{0\text{-}240}$ min for morphine-3-glucuronide was more than three times larger than the $AUC_{0\text{-}240}$ min for morphine. This further supports the role of morphine-3-glucuronide as the dominant metabolite of morphine. The slopes of the elimination phases for morphine and morphine-3-glucuronide were not significantly different ($P > 0.05$), which indicates that morphine-3-glucuronide declines in parallel with morphine during the elimination phase. The result suggests that the elimination rate constant of morphine-3-glucuronide is greater than that of morphine and the formation of morphine-3-glucuronide is the rate-limiting step for the elimination of morphine-3-glucuronide following the administration of morphine [40,41]. The elimination rate constant of morphine-3-glucuronide following direct administration has been determined to be 0.05 min^{-1} [42,43], which is more than twice larger than that of morphine (Table 3).

Normorphine has been identified as a minor metabolite of morphine in several mammalian species including humans and rats [44]. The urinary recoveries of normorphine have been reported to account for 1% and 4% of the administered dose in humans [45] and rats [46], respectively. There is also a pronounced sex difference in the *N*-demethylation of morphine in adult rats [47]. The average microsomal rate of *N*-demethylation of morphine in male rats is 15–22 times greater than that in female rats. The formation of normorphine was rapid after the administration of morphine. The T_{max} of normorphine (9.0 min; Table 3) was not significantly different from that of morphine (8.0 min) ($P > 0.05$). The concentrations of normorphine were much lower than the concentrations of morphine, with the last detectable level at 40 min in all the rats. The $AUC_{0\text{-}40}$ min for normorphine was only

Table 1
Solid-phase extraction recoveries from rat's plasma

Compound	Concentration (nmol l^{-1})	Recovery (%)[a]
Morphine	91	100 ± 3
	895	100 ± 1
Morphine-3-glucuronide	58	82 ± 2
	594	85 ± 2
Morphine-6-glucuronide	63	66 ± 2
	636	63 ± 2
Normorphine	74	89 ± 3
	369	86 ± 4

[a] The results are presented as mean ± standard error of the mean ($n = 6$).

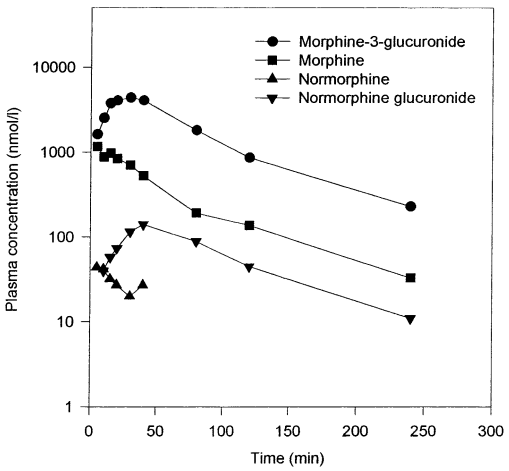

Fig. 2. Representative plasma concentrations versus time curves of morphine-3-glucuronide, morphine, normorphine and normorphine glucuronide from a rat after i.p. administration of morphine at a dose of 14 μmol kg^{-1} (4 mg kg^{-1}).

about 3.4% of the $AUC_{0\text{-}240}$ min for morphine, which is in good agreement with the findings by Klutch [46]. Because of the limited data points, the slope of the elimination phase for normorphine was not calculated.

The 448 > 272 channel was used to monitor the presence of normorphine glucuronide conjugate since the 448 → 272 ion transition indicates the loss of the glucuronic acid moiety. While no peak was detected in the 448 > 272 channel in the blank sample, a peak was consistently detected in the 448 > 272 channel in the samples collected after the administration of morphine. The concentrations corresponding to this compound was estimated from the following equation: $y = a \times x$, where y was the peak area ratio of this compound, a was the response factor for normorphine glucuronide, and x was the calculated concentration. Assuming a similar response factor for normorphine glucuronide as morphine-3-glucuronide relative to morphine, a was thus calculated from the slope of the calibration curve for normorphine

Table 2
Intra- and inter-assay variabilities

Compound	Concentration (nmol l^{-1})	Intra-assay variability (%)[a]	Inter-assay variability (%)[b]
Morphine	91	6.4	4.4
	895	3.8	4.8
Morphine-3-glucuronide	58	9.3	10
	594	4.0	8.4
Morphine-6-glucuronide	63	9.3	10
	636	2.6	11
Normorphine	74	7.4	N.D.[c]
	369	5.4	N.D.[c]

[a] Six samples were analyzed at each concentration.
[b] Five samples were analyzed daily over a 5-day period.
[c] N.D. = not determined.

Table 3
Pharmacokinetic parameters of morphine and its metabolites in rat's plasma after i.p. administration of morphine at a dose of 14 μmol/kg (4 mg/kg) ($n = 5$)[a]

Compound	$AUC^{b}_{0\text{-}240\ min}$ (nmol l^{-1} min^{-1})	C_{max} (nmol l^{-1})	T_{max} (min)	$t_{1/2}$ (min)	K_e (min^{-1})	Slope[c]
Morphine	$5.5 \times 10^4 \pm 3.4 \times 10^3$	1060 ± 133	8.0 ± 2.0	46 ± 2.3	$0.015 \pm 7.3 \times 10^{-4}$	$6.7 \times 10^{-3} \pm 3.2 \times 10^{-4}$
Morphine-3-glucuronide	$2.4 \times 10^5 \pm 4.6 \times 10^4$	2902 ± 629	28 ± 2.0	N.D.[d]	N.D.	$6.0 \times 10^{-3} \pm 1.8 \times 10^{-4}$
Normorphine	$1.9 \times 10^3 \pm 221$	81 ± 12	9.0 ± 2.4	N.D.	N.D.	N.D.
Normorphine glucuronide	$1.1 \times 10^4 \pm 924$	143 ± 20	34 ± 2.4	N.D.	N.D.	N.D.

[a]The results are presented as mean ± standard error of the mean.
[b]AUC for normorphine was calculated from 0 to 40 min.
[c]The values were calculated by linear regression analyses of the last five data points for morphine and the last four data points for morphine-3-glucuronide.
[d]N.D. = not determined.

multiplied by the ratio of the slope of the calibration curve for morphine-3-glucuronide to that of morphine and was equal to 1.1×10^{-3}. The intercept of the equation was assumed to be zero. Although the absolute identity of this compound could not be confirmed due to the lack of synthetic standard, the plasma concentration profile of this compound (Fig. 2) and the ion transition used in MS-MS analyses strongly suggested this compound as the normorphine glucuronide conjugate. In addition, the elution order of this compound relative to normorphine (Fig. 1C), as compared to morphine-3-glucuronide relative to morphine, further supported the above notion. Both normorphine-3-glucuronide and normorphine-6-glucuronide have been identified in humans after the administration of morphine [48]. Like morphine, the normorphine glucuronide conjugate identified in rats is most likely to exist as normorphine-3-glucuronide. In a recent study in rats [49], the presence of normorphine-3-glucuronide was suggested after i.p. administration of normorphine due to the lack of electrochemical signal of this metabolite. The concentrations of normorphine glucuronide were lower than the concentrations of morphine. There is apparently a time-lag for the formation of normorphine glucuronide in three of the five rats because normorphine glucuronide could not be detected at 5 min in the three rats. No normorphine glucuronide could be detected at 240 min in two of the five rats. The $AUC_{0\text{-}240}$ min for normorphine glucuronide was about 20% of the $AUC_{0\text{-}240}$ min for morphine. The slope of the linear portion of the elimination phase could not be accurately determined due to the limited data points available.

In conclusion, a highly sensitive and selective LC-MS-MS assay was developed for the analyses of morphine and its metabolites. By employing only 100 μl of plasma sample, this assay would have value in clinical studies where only limited volumes of samples are available. With its high selectivity, this assay would also be extremely desirable in forensic analyses and its application might be extended to the analyses of other drugs of abuse.

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