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Determination of morphine, morphine-3-glucuronide and morphine-6-glucuronide in monkey and dog plasma by high-performance liquid chromatography–electrospray ionization tandem mass spectrometry

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Abstract

A specific and simultaneous assay of morphine, morphine-3-glucuronide (M-3-G) and morphine-6-glucuronide (M-6-G) in monkey and dog plasma has been developed. These methods are based on rapid isolation using solid phase extraction cartridge, and high-performance liquid chromatography (HPLC)–electrospray ionization (ESI)-tandem mass spectrometric (MSMS) detection. Analytes were separated on a semi-micro ODS column in acetonitrile–formic (or acetic) acid mixed solution. The selected reaction monitoring for assay in monkey and dog plasma, as precursor → product ion combinations of *m/z* 286 → 286 for morphine, *m/z* 462 → 286 for glucuronides and *m/z* 312 → 312 for internal standard (IS, nalorphine) were used. The linearity of morphine, M-3-G and M-6-G was confirmed in the concentration range of 0.5–50, 25–2500, 2.5–250 ng/ml in monkey plasma, 0.5–100, 25–5000, 2.5–500 ng/ml in dog plasma, respectively. The precision of this assay method, expressed as CV, was less than 15% over the entire concentration range with adequate assay accuracy. Therefore, the HPLC–ESI–MSMS method is useful for the determination of morphine, M-3-G and M-6-G with sufficient sensitivity and specificity in pharmacokinetic studies.

Keywords: Morphine; Morphine-3-glucuronide; Morphine-6-glucuronide; Monkey plasma; Dog plasma; HPLC–MSMS

1. Introduction

Morphine (MOR) is the most frequently used opiate analgesic for the treatment of moderate to severe cancer pain. MOR is absorbed from gastrointestinal tract following oral administration, and metabolized by conjugation to morphine-3-glucuronide (M-3-G) and morphine-6-glucuronide (M-6-G) [1]. The analgesic effect of M-6-G are equal to or more potent than MOR [2–5], and M-6-G concentration level in plasma is higher than that of MOR [6]. On the other hand, M-3-G has little analgesic effect [7,8], but M-3-G concentration level in plasma is 5–10 times higher than that of MOR [9] and it may functionally antagonize MOR or M-6-G-induced antinociception and play a role of tolerance to the antinociceptive effects

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of MOR [10]. Therefore, simultaneous determination of MOR and its glucuronides will be required to adequate explain the pharmacokinetics and pharmacodynamics of morphine treatment. For pharmacokinetic study, various assay method have been developed. Radio-immunoassay methods [9] have high sensitivity, but may low specificity by cross-reactivity among MOR, M-3-G and M-6-G. High performance liquid chromatograph (HPLC) methods are widely used, and allow simultaneous analysis of MOR and its glucuronides detected by ultraviolet (UV) detection [11–14], fluorescence (FL) detection [15,16], combined electrochemical detection (ECD)–UV detection [17–19], and combined ECD–FL detection [20,21]. UV and FL detection were not sensitive enough for MOR and its glucuronides in biological samples, and these compounds could be detected by ECD but combined ECD chromatographic consume long time to determine. Mass spectrometric methods [22–26] have achieved the desired sensitivity and selectivity. The HPLC–mass spectrometry–mass spectrometry (MSMS) method [27–30] has recently been demonstrated to be a useful technique for a rapid quantitative determination, however, to our knowledge, HPLC–MSMS method for analysis of MOR, M-3-G and M-6-G in monkey and dog plasma has not been reported. For preliminary study of new characteristic formulation of morphine such as extended release tablet, the determination method in monkey or dog is very useful, because it is definitely difficult to use narcotic compounds. In this paper, we report the selective, sensitive and simultaneous determination of MOR, M-3-G and M-6-G in monkey and dog plasma by HPLC–MSMS with selected reaction monitoring (SRM), and its application in pharmacokinetic study.

2. Experimental

2.1. Reagents and materials

Morphine sulfate·$5H_2O$ was from Sankyo (Tokyo, Japan) and morphine-3-glucuronide and morphine-6-glucuronide were from Sigma-Aldrich (St. Louis, MO, USA). Nalorphine hydrochloride obtained from RBI (Natick, MA, USA) was used as an internal standard (IS). Sodium carbonate anhydrous, hydrochloric acid, acetic acid and formic acid were of reagent grade from Katayama Kagaku (Osaka, Japan). Methanol and acetonitrile were of HPLC grade from Fisher (Fair Lawn, NJ, USA). OASIS HLB 1 cc (30 mg) extraction cartridges were obtained from Waters (Milford, MA, USA). Argon (99.9999%) was purchased from Taiyo Toyo Sanso (Osaka, Japan).

2.2. Standard solutions

The stock standard solutions of MOR, M-3-G and M-6-G (100 μg/ml) were prepared with distilled water. This solution was further diluted with distilled water to the working standard solutions at given concentrations for validation and calibration. The stock IS solution (100 μg/ml) was also prepared as the stock standard solution with methanol. The working IS solution of 1 μg/ml was prepared by dilution of the stock IS solution with distilled water. All stock solutions and working solutions were stored at 4 °C.

Sodium carbonate buffer (pH 9, 0.005 M) was prepared by dissolving 0.53 g of sodium carbonate anhydrous in 1000 ml of distilled water, adjusting pH to 9 with hydrochloric acid. Sodium carbonate buffer (pH 10, 0.2 M) was prepared by dissolving 21.2 g of sodium carbonate anhydrous in 1000 ml of distilled water, adjusting pH to 10 with hydrochloric acid.

2.3. HPLC–MSMS

A TSQ 7000 tandem mass spectrometer (Thermo Finnigan, San Jose, CA, USA) equipped with an electrospray ionization (ESI) interface, a nitrogen generator (System Instruments, Tokyo, Japan) and a 2690 Separation Module (Waters, Milford, MA, USA) was used for all HPLC–MSMS analyses.

HPLC separation was performed using Symmetry C_{18} (Waters, Milford, MA, USA), 2.1 mm i.d. × 150 mm, 5 μm particle size, at a column temperature of 40 °C. Acetonitrile–0.05% (v/v) acetic acid (1:24, v/v) was used as mobile phase for monkey plasma, and methanol–0.01% (v/v) formic acid (1:19, v/v) was used as mobile phase for dog plasma. The injection volume was 10 μl, run time was 9 min, the flow rate was 0.1 ml/min, and the temperature of the sample cooler in auto-sampler was set at 20 °C.

A TSQ 7000 was operated in the positive-ion mode at following conditions: nitrogen (>99%) was used for the sheath gas and auxiliary gas at pressures of

60 psi and 5 units, respectively. The temperature of the heated capillary was maintained at 170 °C, and the spray voltage of ESI was set at 4.5 kV. A collision-induced dissociation (CID) was achieved using argon as the collision gas at the pressure adjusted to more than 2.8 mTorr above normal, and the applied collision offset energy was set to −30 eV. The electron multiplier voltage and dynode voltage were run at 1500 V and 15 kV, respectively. The data was acquired at the scan rate of 3 s for all scans. The ions for SRM analysis of MOR, M-3-G, M-6-G and IS were selected at *m/z* 286, 462, 462, and 312 as the precursor ion set mass in the first quadrupole, and *m/z* 286, 286, 286, and 312 as product ion set mass in the third quadrupole, respectively. The MSMS system was programmed for SRM, that is, the acquisition was programmed by the unique programming language of this system for 2.0–8.5 min after the sample injection.

2.4. Sample preparation

A 0.2 ml aliquot of plasma sample was pipetted into a glass test tube, and 0.1 ml of distilled water (or working standard solution for calibration), 0.1 ml of working IS solution, and 0.5 ml of sodium carbonate buffer (pH 10, 0.2 M) were added. The mixture was applied to an OASIS HLB cartridge, which was previously conditioned with 1 ml of methanol, 1 ml of distilled water, and 1 ml of sodium carbonate buffer (pH 10, 0.2 M), respectively. The cartridge was washed with 1 ml of sodium carbonate buffer (pH 9, 0.005 M) and 1 ml of distilled water.

MOR, M-3-G, M-6-G and IS retained in the cartridge were eluted with 0.5 ml of methanol into a disposable glass test tube, and evaporated to dryness at 40 °C under a stream of nitrogen. The residue was dissolved in 0.2 ml of the mobile phase (see Section 2.3 HPLC–MSMS), and 10 μl of that was analyzed by HPLC–MSMS using SRM technique in product ion scan mode.

2.5. Validation tests

2.5.1. Linearity and calibration curve

Standard samples for linearity at eight concentrations in monkey plasma, MOR; 0, 0.5, 1, 2.5, 5, 10, 25 and 50 ng/ml, M-3-G; 0, 25, 50, 125, 250, 500, 1250 and 2500 ng/ml, M-6-G; 0, 2.5, 5, 12.5, 25, 50, 125 and 250 ng/ml were prepared and assayed. To determine the precision of the slope of the calibration curve, each calibration standard at six concentrations, MOR; 0, 0.5, 1, 5, 25 and 50 ng/ml, M-3-G; 0, 25, 50, 250, 1250 and 2500 ng/ml, M-6-G; 0, 2.5, 5, 25, 125 and 250 ng/ml, in monkey plasma were also prepared and assayed.

Standard samples for linearity at nine concentrations in dog plasma, MOR; 0, 0.5, 1, 2, 5, 10, 25, 50 and 100 ng/ml, M-3-G; 0, 25, 50, 100, 250, 500, 1250, 2500 and 5000 ng/ml, M-6-G; 0, 2.5, 5, 10, 25, 50, 125, 250 and 500 ng/ml were prepared and assayed. To determine the precision of the slope of the calibration curve, each calibration standard at six concentrations, MOR; 0, 0.5, 2, 10, 50 and 100 ng/ml, M-3-G; 0, 25, 100, 500, 2500 and 5000 ng/ml, M-6-G; 0, 2.5, 10, 50, 250 and 500 ng/ml, in dog plasma were also prepared and assayed.

A linear model was fit to the concentration versus peak-area ratio data using the least squares regression, and the concentration of 0 was not used for the calibration curve.

2.5.2. Specificity and interference

Chromatograms of the sample prepared with monkey or dog blank plasma were visually inspected for peaks from endogenous sources which might correspond to MOR, M-3-G, M-6-G and IS peaks.

2.5.3. Accuracy and precision

Samples at each of three concentrations, MOR; 0.5, 5 and 50 ng/ml, M-3-G; 25, 250 and 2500 ng/ml, M-6-G; 2.5, 25 and 250 ng/ml in monkey plasma ($n = 6$, respectively), were prepared and assayed to determine the intra- or inter-day accuracy expressed as relative error (RE), and precision as coefficient of variation (CV). For dog plasma, MOR; 0.5, 10 and 100 ng/ml, M-3-G; 25, 500 and 5000 ng/ml, M-6-G; 2.5, 50 and 500 ng/ml ($n = 6$, respectively) were prepared and assayed.

2.5.4. Stability

Analyte stability was tested through three freeze-thaw cycles, and also at −20 °C in the freezer for a long period. Post-extraction analyte stability in autosampler at 20 °C was determined by comparing the results to those of freshly extracted samples.

3. Results and discussion

3.1. HPLC–MSMS conditions

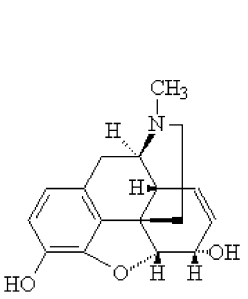
3.1.1. HPLC

It is necessary to separate both peak of M-3-G and M-6-G completely on HPLC since M-3-G and M-6-G are isomers each other even if mass spectrometric detection is used, because they have the same molecular weights, 461 as shown in Fig. 1. After several types of HPLC columns were investigated it was found Symmetry C_{18} column could rapidly separate these hydrophilic compounds on HPLC with acetonitrile–0.05% (v/v) acetic acid (1:24, v/v, for monkey) or methanol–0.01% (v/v) formic acid (1:19, v/v, for dog). Using mobile phase for monkey, an unknown peak originated from dog plasma was found and disturbed the determination of morphine and its metabolite, however no interference was observed using methanol-based mobile phase.

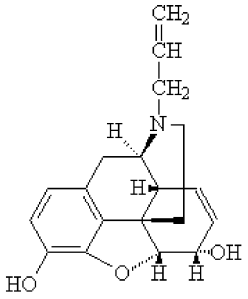
3.1.2. MSMS

Each compound was first directly introduced in a mass spectrometer using the loop injector and ESI interface to get individual mass spectra. Parameters such as the temperature of the heated-capillary, spray voltage, flow of sheath-gas and auxiliary-gas were optimized in order to obtain much stronger intensity of the protonated molecule. In ESI-positive mass spectra of each compounds, the protonated molecule $[M + H]^+$ was identified at *m/z* 286, 462, 462 and 312 for MOR, M-3-G, M-6-G and IS, respectively. In single MS chromatogram using these protonated molecule ions, a small interference peak at retention time of MOR was observed, and then MSMS detection was applied for the determination of MOR, M-3-G and M-6-G.

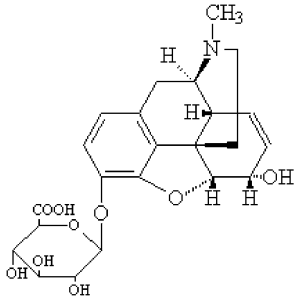
As shown in Fig. 2, product-ion spectra of MOR, M-3-G, M-6-G and IS were acquired with these protonated molecules as precursors. Predominant fragment ions, *m/z* 286, were detected with strong intensity in spectra of M-3-G and M-6-G, therefore the mass set



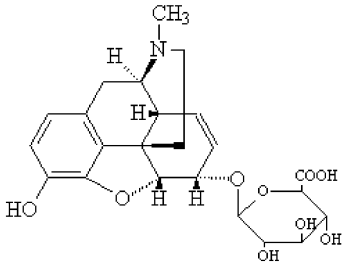
Morphine : $C_{17}H_{19}NO_3$



Nalorphine : $C_{19}H_{21}NO_3$



Morphine-3-glucuronide : $C_{23}H_{27}NO_9$



Morphine-6- glucuronide : $C_{23}H_{27}NO_9$

Fig. 1. Chemical structures of MOR, M-3-G, M-6-G and nalorphine (IS).

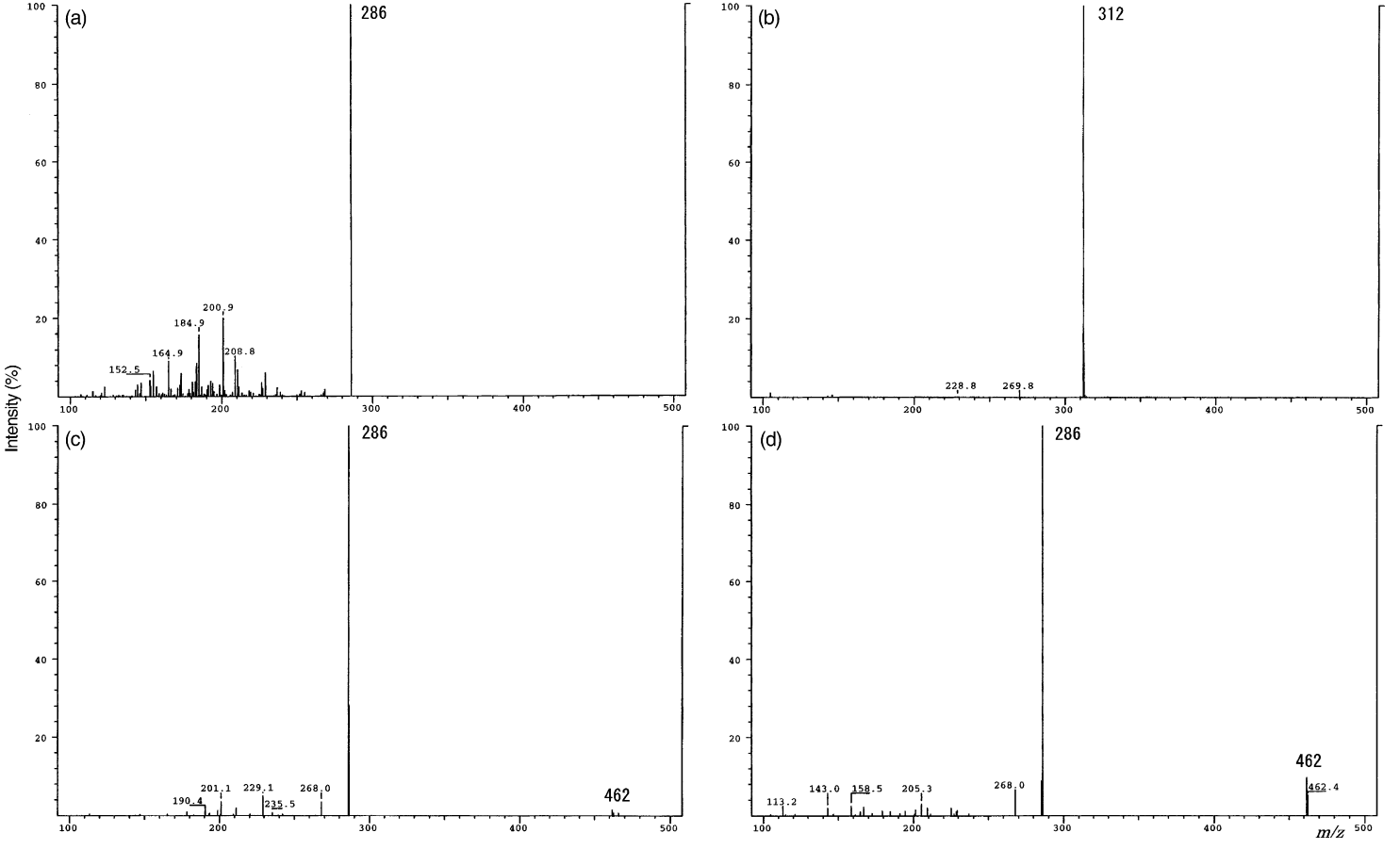


Fig. 2. MSMS spectra of MOR, M-3-G, M-6-G and nalorphine (IS). (a) MOR, (b) IS, (c) M-3-G, (d) M-6-G.

of *m/z* 462 and *m/z* 286 was selected for SRM monitoring. On the other hand, some small fragment ions were detected in spectra of MOR and IS and the intensity of protonated ion was absolutely larger than that of fragment ions. The selection of the same ion for precursor and product ions was very effective for disappearance of the interference peak without loss of sensitivity in this case. It seems that the interference originated from plasma at *m/z* 286 is easily dissociated in collision cell by setting conditions, but the molecule of MOR and IS is affected very little. Therefore, the ion set (precursor to product) for SRM were selected at *m/z* 286 → 286 for MOR, *m/z* 462 → 286 for M-3-G and M-6-G, and *m/z* 312 → 312 for IS, respectively.

3.2. Sample preparation

A highly sensitive and reproducible analytical method for biological samples needs suitable, sometimes tedious, pre-treatments if low selective detection system was used. The technique using MSMS has high selectivity and specificity, and is considered to simplify the preparation procedure to the greatest extent possible.

The OASIS HLB solid phase extraction (SPE) cartridge was chosen to isolate MOR, M-3-G and M-6-G from monkey and dog plasma. The plasma sample on basic condition with sodium carbonate buffer was charged into the previously conditioned OASIS HLB cartridge. It is not necessary to worry that the SPE cartridge was packed by plasma components because of low volume of plasma sample used. After washing the cartridge, MOR, M-3-G and M-6-G were isolated by elution with methanol. These procedures make it possible to extract MOR and its glucuronides from monkey and dog plasma rapidly and quantitatively.

3.3. Validation

3.3.1. Linearity and calibration curves

Good linearity was observed over the concentration range of MOR; 0.5–50 ng/ml ($r^2 = 0.9998$), M-3-G; 25–2500 ng/ml ($r^2 = 0.9955$), M-6-G; 2.5–250 ng/ml ($r^2 = 0.9998$) in monkey plasma, and MOR; 0.5–100 ng/ml ($r^2 = 1.0000$), M-3-G; 25–5000 ng/ml ($r^2 = 0.9992$), M-6-G; 2.5–500 ng/ml ($r^2 = 0.9998$) in dog plasma.

3.3.2. Selectivity and specificity

HPLC–MSMS in the SRM mode provides a highly selectivity for the determination of compounds in biological samples. The representative SRM chromatograms of blank monkey and dog plasma and spiked plasma samples are shown in Figs. 3 and 4. No endogenous sources of interference were observed at the retention time of analytes obtained from six sources of blank plasma.

3.3.3. Accuracy, precision and limit of quantification

The intra- and inter-day accuracy and precision are assessed in Tables 1 and 2. The error % of MOR, M-3-G and M-6-G in monkey plasma was ranged from −8.2 to 2.0%, −1.9 to 7.5% and −12.5 to 2.1% for intra-day, and −4.1 to 4.1%, 0.9 to 10.2% and −8.3 to −0.5% for inter-day, respectively. The CV of them ranged from 5.3 to 18.5%, 5.3 to 11.6%, and 2.8 to 5.4% for intra-day, and 7.7 to 9.4%, 4.9 to 9.9%, and 4.3 to 6.0% for inter-day, respectively. On the other hand, in dog plasma, the error % of MOR, M-3-G and M-6-G ranged from −4.0 to 0.0%, −0.2 to 16.5% and −3.4 to 4.3% for intra-day, and −1.4 to 1.0%, 2.6 to 18.5% and −9.5 to 4.3% for inter-day, respectively. The CV of them ranged from 4.1 to 9.7%, 3.8 to 6.5%, and 3.5 to 7.5% for intra-day, and 4.7 to 7.9%, 7.7 to 10.1%, and 6.3 to 18.0% for inter-day, respectively.

The limit of quantification of MOR, M-3-G and M-6-G was established at 0.5, 25, 2.5 ng/ml in monkey and dog plasma on the basis of the accuracy of the determinations at this concentration (deviation from the nominal value within 20%).

3.4. Stability

The stability tests were designed to cover the anticipated conditions that samples of pharmacokinetic study may experience. Stabilities of MOR, M-3-G and M-6-G at −20 °C in plasma, at 20 °C in extracted sample, and after freeze-thaw cycles were tested and established. The results were summarized in Table 3. These compounds in monkey and dog plasma after three freeze-thaw cycles showed remaining as the percentage is more than 94% compared with initial concentration found. Analytes in monkey and dog plasma remained stable in a freezer at −20 °C for 78 days in monkey plasma and for 90 days in dog plasma.

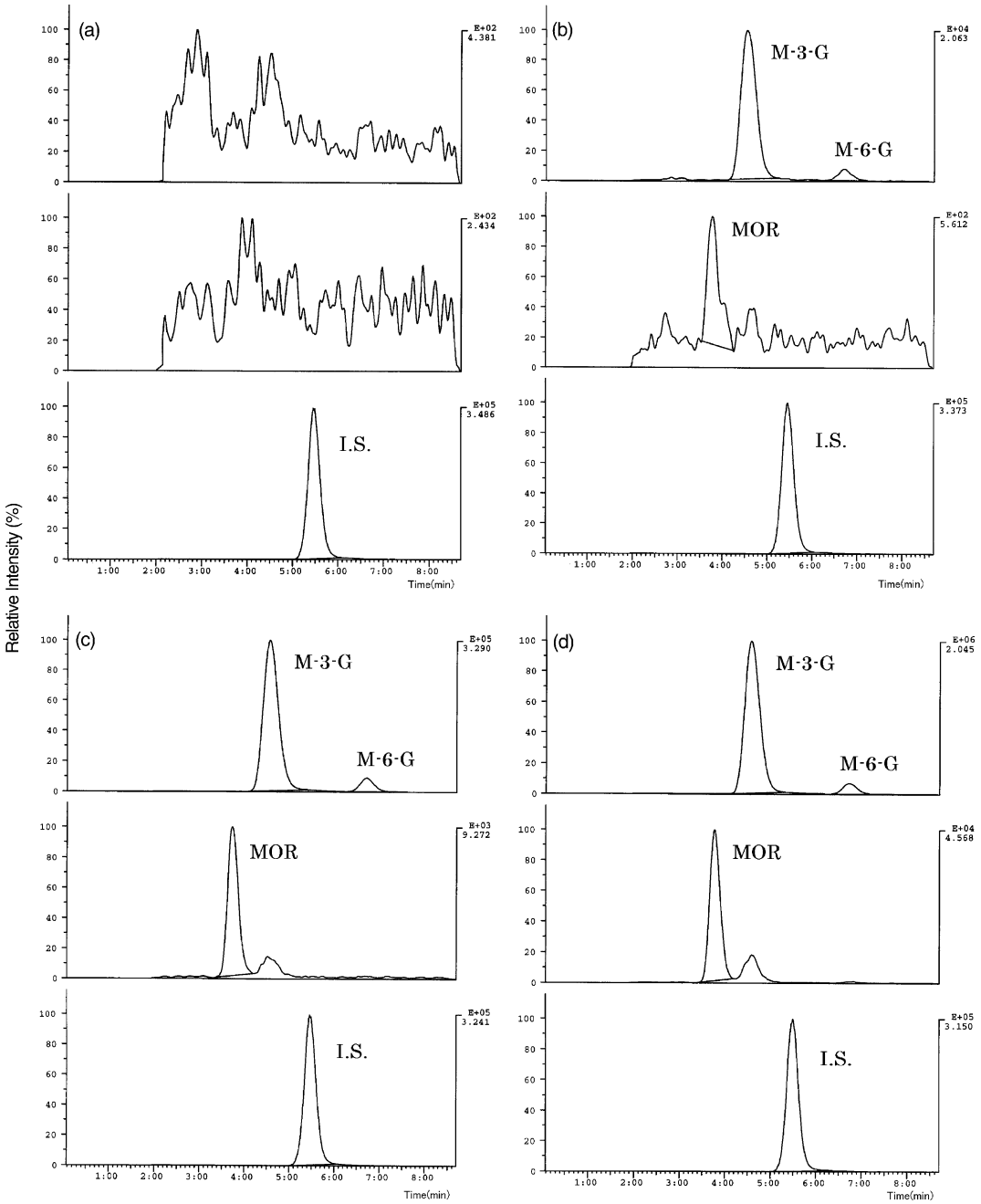


Fig. 3. Representative HPLC–MSMS chromatograms of MOR, M-3-G, M-6-G and IS in monkey plasma. (a) Blank plasma with IS, (b) MOR: 0.5 ng/ml, M-3-G: 25 ng/ml, M-6-G: 2.5 ng/ml, (c) MOR: 10 ng/ml, M-3-G: 500 ng/ml, M-6-G: 50 ng/ml, (d) MOR: 50 ng/ml, M-3-G: 2500 ng/ml, M-6-G: 250 ng/ml.

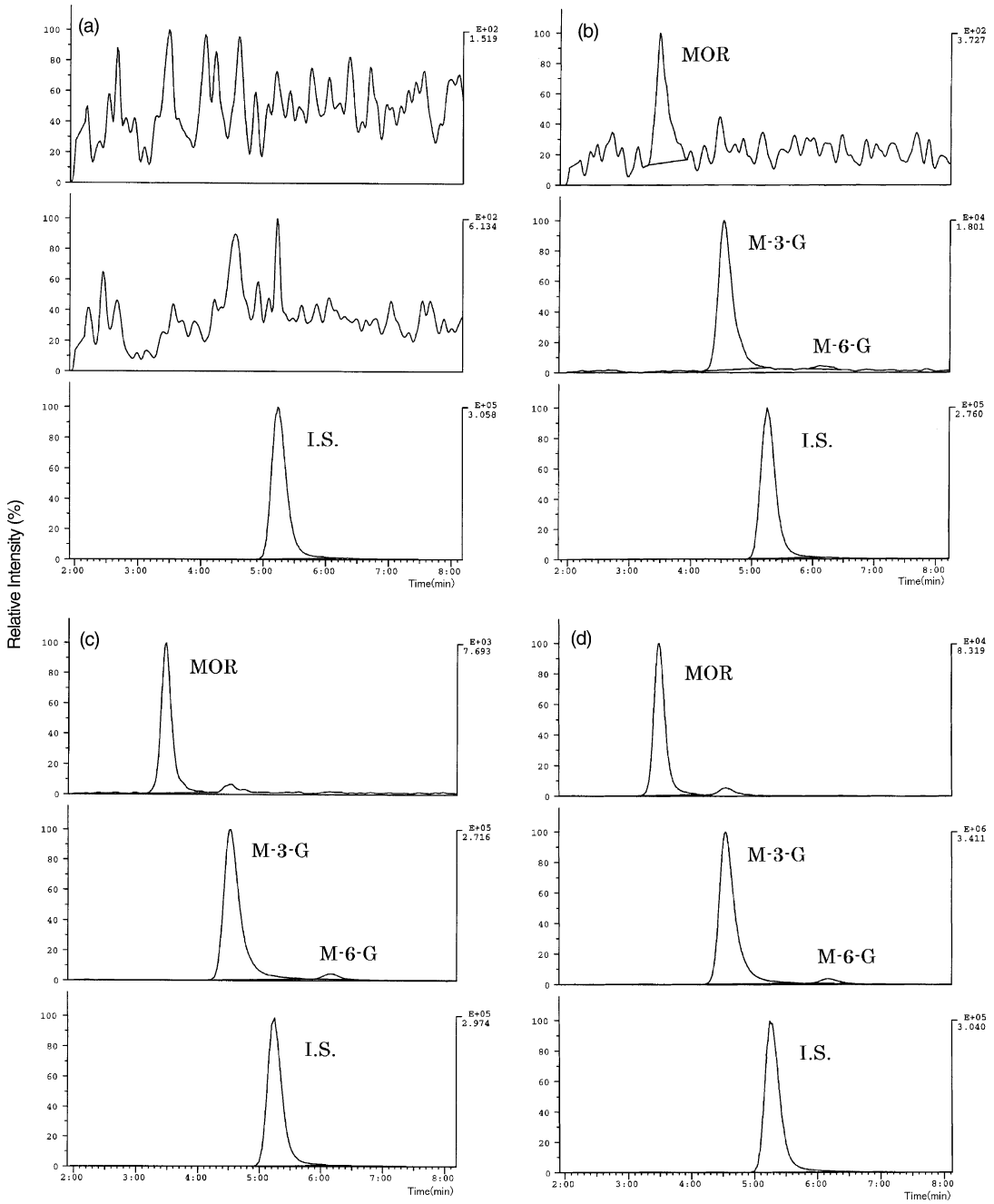


Fig. 4. Representative HPLC–MSMS chromatograms of MOR, M-3-G, M-6-G and IS in dog plasma. (a) Blank plasma with IS, (b) MOR: 0.5 ng/ml, M-3-G: 25 ng/ml, M-6-G: 2.5 ng/ml, (c) MOR: 10 ng/ml, M-3-G: 500 ng/ml, M-6-G: 50 ng/ml, (d) MOR: 100 ng/ml, M-3-G: 5000 ng/ml, M-6-G: 500 ng/ml.

Table 1
Intra-day precision and accuracy of MOR, M-3-G and M-6-G spiked in monkey and dog plasma by HPLC–MSMS ($n = 6$)

	Compound	Nominal concentration (ng/ml)	Found (mean ± S.D.) (ng/ml)	Precision (CV, %)	Accuracy (RF, %)
Monkey	MOR	0.49	0.50 ± 0.09	18.5	2.0
		4.9	4.5 ± 0.2	5.3	−8.2
		49.1	48.2 ± 5.9	12.3	−1.8
	M-3-G	25.6	26.9 ± 2.2	8.1	5.1
		256.0	251.1 ± 29.1	11.6	−1.9
		2560	2751.8 ± 144.8	5.3	7.5
	M-6-G	2.4	2.1 ± 0.1	3.0	−12.5
		24.1	22.8 ± 1.2	5.4	−5.4
		240.9	245.9 ± 6.9	2.8	2.1
Dog	MOR	0.5	0.5 ± 0.1	9.7	0.0
		10.1	97 ± 0.4	4.2	−4.0
		101.0	98.7 ± 4.1	4.1	−2.3
	M-3-G	25.4	29.6 ± 1.2	3.9	16.5
		507.5	532.4 ± 24.5	4.6	4.9
		5075	5064.5 ± 194.4	3.8	−0.2
	M-6-G	2.3	2.4 ± 0.2	7.5	4.3
		46.6	45.5 ± 1.6	3.5	−2.4
		465.7	449.8 ± 18.6	4.1	−3.4

Table 2
Inter-day precision and accuracy of MOR, M-3-G and M-6-G spiked in monkey and dog plasma by HPLC–MSMS ($n = 6$)

	Compound	Nominal concentration (ng/ml)	Found (mean ± S.D.) (ng/ml)	Precision (CV, %)	Accuracy (RF, %)
Monkey	MOR	0.49	0.51 ± 0.04	8.0	4.1
		4.9	4.7 ± 0.4	9.4	−4.1
		49.1	47.5 ± 3.7	7.7	−3.3
	M-3-G	25.6	28.2 ± 1.4	4.9	10.2
		256.0	262.4 ± 25.9	9.9	2.5
		2560	2583.7 ± 210.2	8.1	0.9
	M-6-G	2.4	2.2 ± 0.1	6.0	−8.3
		24.1	23.2 ± 1.0	4.3	−3.7
		240.9	239.8 ± 10.9	4.5	−0.5
Dog	MOR	0.5	0.5 ± 0.0	7.9	0.0
		10.1	10.2 ± 0.6	6.3	1.0
		101.0	99.6 ± 4.7	4.7	−1.4
	M-3-G	25.4	30.1 ± 2.8	9.2	18.5
		507.5	540.9 ± 54.6	10.1	6.6
		5075	5204.9 ± 400.3	7.7	2.6
	M-6-G	2.3	2.4 ± 0.4	18.0	4.3
		46.6	42.4 ± 4.9	11.5	−9.0
		465.7	421.5 ± 26.7	6.3	−9.5

Table 3
Stability of MOR, M-3-G and M-6-G in plasma and extracted sample ($n = 3$)

	MOR				M-3-G				M-6-G			
	Monkey		Dog		Monkey		Dog		Monkey		Dog	
	Concentration (ng/ml)	Remained (%)	Concentration (ng/ml)	Remained (%)	Concentration (ng/ml)	Remained (%)	Concentration (ng/ml)	Remained (%)	Concentration (ng/ml)	Remained (%)	Concentration (ng/ml)	Remained (%)
Plasma	4.9	100.0	2.2	105.0	256.0	101.7	112.9	112.9	24.1	97.5	10.4	94.6
Freeze-thaw cycles	39.3	111.4	10.3	112.4	2048	98.2	517.7	112.6	192.7	97.4	47.5	106.8
(3 cycles)			81.3	112.5			4083	108.9			374.7	108.1
Plasma at −20 °C	4.9	88.0	2.2	110.0	256.0	99.1	112.9	99.9	24.1	90.8	10.4	95.7
Monkey: 78 days	39.3	86.2	10.3	114.4	2048	89.7	517.7	97.2	192.7	100.9	47.5	88.2
Dog: 90 days			81.3	108.3			4083	93.6			374.7	90.3
Extracted sample at 20 °C	4.9	112.0	1.0	111.1	256.0	105.9	50.8	94.1	24.1	103.4	4.7	90.7
Monkey: 48 h	39.3	104.1	10.1	98.9	2048	99.0	507.5	95.5	192.7	102.6	46.6	88.4
Dog: 72 h			101.0	92.1			5075	98.0			465.7	91.2

Remained (%): percentage compared with the initial concentration found.

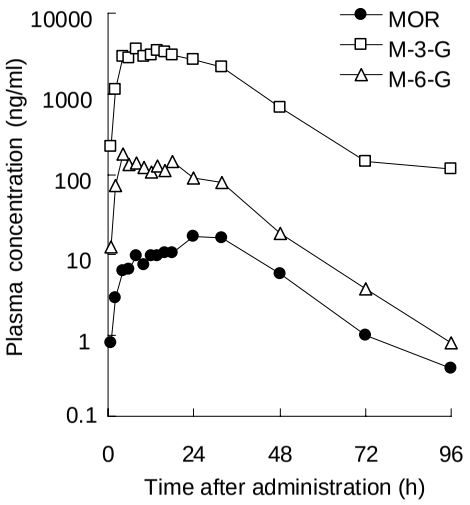


Fig. 5. Plasma concentrations vs. time curves of MOR, M-3-G and M-6-G after oral administration of 120 mg extended release tablet of morphine sulfate to monkeys ($n = 6$).

Post-extraction analyte stability in auto-sampler at 20 °C was assessed. MOR, M-3-G and M-6-G in prepared sample solution after extraction were stable for approximately 48 h for monkey plasma and 72 h for dog plasma, since the found of these compounds were within 99.0–112.0% for monkey plasma, within 88.4–111.1% for dog plasma compared with initial found.

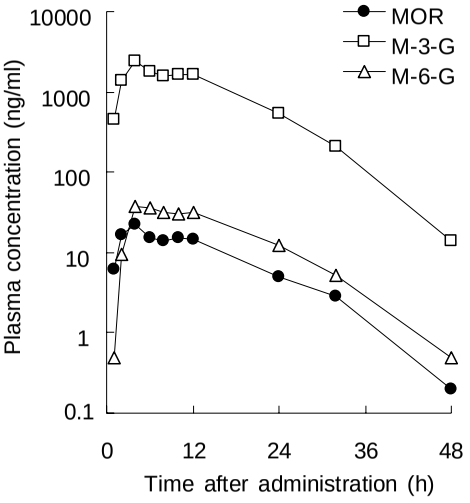


Fig. 6. Plasma concentrations vs. time curves of MOR, M-3-G and M-6-G after oral administration of 120 mg extended release tablet of morphine sulfate to dogs ($n = 6$).

3.5. Application

The present method was applied to the determination of MOR, M-3-G and M-6-G in monkey and dog plasma sample of pharmacokinetic study. The plasma concentration versus time profiles after oral administration of 120 mg extended release tablet of MOR sulfate to monkeys and dogs are shown in Figs. 5 and 6. Plasma concentrations of M-3-G and M-6-G were approximately 200 and 10 times higher than that of MOR in monkeys, and approximately 100 and 2 times higher in dogs, respectively.

4. Conclusions

The HPLC–MSMS method described in this paper permits the simultaneous determination of MOR, M-3-G and M-6-G in monkey and dog plasma with high sensitivity and specificity. The method is suitable for use in pharmacokinetic studies, because it needs only a small amount of plasma sample to determine MOR, M-3-G and M-6-G. By using solid phase extraction and SRM monitoring it enables to determine these analytes rapidly, and no deconjugation of M-3-G and M-6-G was observed during sample preparation. Therefore, the HPLC–MSMS method is useful for the determination of MOR, M-3-G and M-6-G with sufficient selectivity and sensitivity in pharmacokinetic studies of monkeys and dogs, and the development of unique characteristic formulation.

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