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Rapid and simple method to determine morphine and its metabolites in rat plasma by liquid chromatography–mass spectrometry☆

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Abstract

A rapid and simple method for the determination of morphine (M), normorphine (NM), morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G) in plasma by high-performance liquid chromatographic separation with mass spectrometric detection (HPLC–MS) has been developed. Samples (40 μl) were cleaned-up by protein precipitation with two volumes (80 μl) of acetonitrile and reconstituted in formic acid 0.1% in water. Naloxone was used as internal standard. Analytes were separated on a phenyl–hexyl column using a step-gradient (1 ml/min) of acetonitrile and formic acid in water. Acetonitrile was added post-column (0.3 ml/min). Quantification of morphine and its metabolites was achieved with an Agilent 1100 series HPLC–MS system equipped with electrospray interface set to selected ion-monitoring (SIM) mode. Calibration curves covered a wide range of concentrations (2.44–10 000 n*M*) and were best fitted with a weighed quadratic equation. The limits of quantification achieved with this method were 2.44 n*M* for M and 4.88 n*M* for NM, M3G and M6G. The method proved accurate (85–98%), precise (C.V.<10%) and was successfully applied to a wide range of in vitro and in vivo pharmacokinetic studies in rodents.
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Keywords: Morphine; Normorphine; Morphine glucuronides

1. Introduction

Morphine (M) is a μ-opioid agonist traditionally used for the treatment of moderate to severe pain [1]. It is extensively metabolized to its morphine-3-glucuronide (M3G), with normorphine (NM) and morphine-6-glucuronide (M6G) as minor metabolites (Fig. 1), irrespective of the species or administration route [2–4]. The extent of formation of M metabolites, however, differs among species [2,5]. Compared to humans, rats do not produce detectable amounts of M6G while they are able to form more NM [2,6]. In vitro, M6G and NM possess high affinity for the μ-opioid receptor, although this affinity is not higher than that of morphine [7]. In vivo, M-6-G and NM are analgesic [8–12] and may

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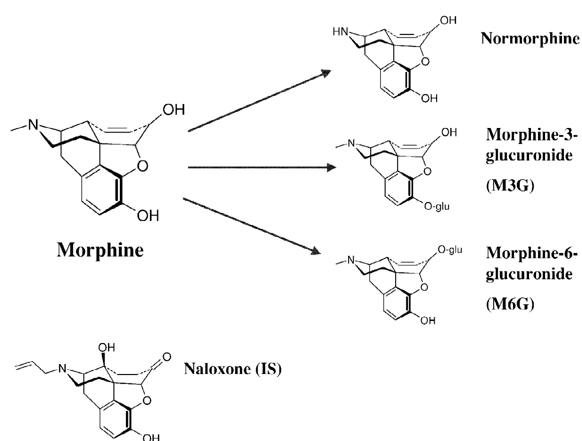

Fig. 1. Chemical structures of morphine (M) and its metabolites, morphine-3-glucuronide (M3G), morphine-6-glucuronide (M6G) and normorphine (NM).

therefore contribute to morphine-induced analgesia [13] or side effects [14], especially following long-term oral treatment. In contrast, M3G is devoid of antinociceptive properties and has been shown to antagonize morphine-induced analgesia in animal models [15].

Morphine is routinely used as a reference compound in drug discovery programs targeting new analgesics. In order to draw pharmacokinetic/pharmacodynamic (PK/PD) relationships and/or understand the impact of various disease states on PK, it is of interest to measure plasma concentrations of both M and its metabolites. Consequently fast, easy and reliable analytical methods are required to ensure adequate turnaround times. High sensitivity is also essential as sample volumes are very limited by the use of rodents for in vivo studies.

Various methods have been developed for the simultaneous determination of M and its metabolites in biological fluids. Immunological assays lack specificity between morphine and its metabolites. Their usefulness is therefore limited and lead to complicated data analyses when quantitative results are needed [16]. Several reversed-phase high-performance liquid chromatography (RP-HPLC) methods have been described and report the separation of M and its metabolites using ultraviolet (UV) [17,18], fluorescence [19–21] or electrochemical [22,23] detections. UV suffers from lack of sensitivity, often precluding the accurate description of the later part of the plasma concentration versus time curves. Electrochemistry may be very sensitive but M3G cannot be quantified by this method since it lacks an oxidizable phenolic group. Gas chromatography coupled to mass spectrometry (MS) [24,25], may offer adequate sensitivity but requires time-consuming sample preparation and derivatization procedures. Furthermore, morphine glucuronides have to be measured indirectly as total glucuronides, which results in a loss of precious information.

Recently, several reports have described analysis of M and its metabolites by RP-HPLC–MS [26–28] or RP-HPLC–tandem MS (MS–MS) [29–32] coupled to atmospheric pressure-electrospray ionization (AP-ESI). In spite of improved selectivity and sensitivity, the systems require highly qualified technical expertise (for tandem-MS) or extensive sample clean up relying mainly on solid-phase or liquid–liquid extraction for optimal detector response. In order to provide robust and accurate data in a timely manner, we have developed and validated a new HPLC–MS assay that allows the determination of M and its major metabolites in rat plasma without any solid-phase or liquid–liquid extraction.

2. Experimental

2.1. Chemicals and reagents

Morphine sulfate pentahydrate and formic acid were obtained from BDH (Toronto, Ontario, Canada). Normorphine hydrochloride and morphine-3-glucuronide were purchased from Sigma–Aldrich (St-Louis, MO, USA). Morphine-6-glucuronide was obtained from Lipomed (Cambridge, MA, USA). The internal standard (I.S.), naloxone hydrochloride, was purchased from RBI (Natick, MA, USA). HPLC grade acetonitrile and formic acid were obtained from VWR International (Montréal, Québec, Canada). Purified water was obtained using a Nano-Pure water purification system (Barnstead, Dubuque, IA, USA).

2.2. Preparation of stock solutions, calibration standards and quality control samples

Stock solutions (5 m*M*) of M3G, M6G, M, NM and the I.S. naloxone were prepared in purified water. Naloxone was diluted in 0.1% formic acid in

acetonitrile to a final concentration of 1 μ*M*. "Individual working solutions" (10 μ*M*) of M, NM, M3G, M6G and naloxone were prepared by diluting stock solutions in 0.1% formic acid for optimization of chromatographic and MS conditions. M, NM, M3G and M6G stock solutions were combined and diluted with water to obtain a "pooled working solution" containing 1 m*M* of each of the four analytes. All solutions were stored in amber glass tubes and kept at −20 °C. Analytes did not interfere with each other and standard curves obtained with individual analytes were comparable with that obtained with the pooled solution.

Calibration standards and quality control (QC) samples were prepared on the same day by spiking appropriate amounts of two different "pooled working solutions" in drug free EDTA plasma obtained from Sprague–Dawley rats. The calibration curves were constructed using 12 or 13 concentration points. For M, concentration points were 2.44, 4.88, 9.77, 19.5, 39.1, 78.1, 156, 312, 625, 1250, 2500, 5000 and 10 000 n*M*. For NM, M3G and M6G, concentration points were 4.88, 9.77, 19.5, 39.1, 78.1, 156, 312, 625, 1250, 2500, 5000 and 10 000 n*M*.

These ranges cover the plasma concentrations expected in our experimental studies. Calibration curves, which relate the analytes concentration to the peak area ratio of analytes over the I.S. were best fitted with a quadratic equation weighed with $1/x$ factor. A quadratic regression was used for quantification since it produces a more accurate fit over a larger dynamic range compared to linear regression. QC sample concentrations covered lower (10 n*M*), medium (400 n*M*) and higher (4000 n*M*) ranges of the standard curves. All QC and standards were kept at −80 °C.

2.3. HPLC–AP-ESI–MS analysis

The assay was performed using an Agilent 1100 series HPLC–MS system (Agilent technologies, Ville St-Laurent, Canada). Liquid chromatographic separations were achieved using a Luna 3 μm phenyl–hexyl column (4.6×75 mm) that was preceded by a phenyl–hexyl 3 μm guard column (4.6×30 mm) (Phenomenex, Torrance, CA, USA). The column temperature was kept constant at 40 °C. The mobile phase consisted of a mixture of 0.1% formic acid in water (A) with 0.1% formic acid in acetonitrile (B) and was delivered at a flow-rate of 1 ml/min. A step-wise gradient of B into A was run over 5.5 min, after 3.5 min with A in isocratic mode. It consisted of a linear gradient increasing to 5% of B over 1.5 min, followed by a rapid increase to 70% of B over 4 min. A re-equilibration phase of 3 min was allowed between samples. An isocratic pump (Agilent technology, Ville St-Laurent, Canada) was programmed to deliver acetonitrile post-column at 0.3 ml/min via a post-column addition tee. The total flow-rate delivered to the MS source was 1.3 ml/min.

The single quadrupole MS was equipped with an AP-ESI source and operated in selected ion-monitoring (SIM) mode. The protonated quasi-molecular ions were used to quantify NM (m/z=272), M (m/z=286), M3G and M6G (m/z=462). MS parameters were set to facilitate the ionization process and achieve the best sensitivity. In order to minimize contamination of the AP-ESI source from potential endogenous interferences coming from the sample matrix, a switching-valve strategy was implemented. The LC flow was directed to the waste for the first 3 min following the injection before entering the source. The nebulizer pressure was set at 60 p.s.i.g., while the drying gas (nitrogen) was delivered at a flow-rate of 13 l/min at a temperature of 350 °C. Capillary voltage was set at 3.5 kV and the fragmentor (collision-induced dissociation cell) was set at 120 V from 3 to 7.2 min and at 150 V from 7.2 to 9 min. These conditions allowed minimal fragmentation of the parent compound, optimal yield of its quasi-molecular ion and minimized interferences from the matrix. Chromatograms were integrated using the HP ChemStation software package (Rev 8.04) (Agilent technologies, Ville St-Laurent, Canada).

2.4. Sample preparation

Plasma samples were vortex-mixed briefly and aliquots (40 μl) were transferred to microcentrifuge tubes. Proteins were precipitated by the addition of two sample volumes (80 μl) of ice-cold acetonitrile containing the I.S. (1 μ*M*). Following precipitation, tubes were vortex-mixed and centrifuged at 16 000 *g* for 10 min. Supernatants (100 μl) were evaporated to dryness under a stream of nitrogen. Dry residues were reconstituted with 40 μl of 0.1% formic acid,

centrifuged for 10 min at 16 000 *g* and aliquoted into 96-well plates. The plate was transferred to a refrigerated (4 °C) autosampler and 3–20 μl of samples were injected into the HPLC–MS system.

2.5. Assay validation

2.5.1. Sensitivity and specificity

The limit of quantitation (LOQ) was determined as the minimum concentration that could be accurately and precisely quantified (lowest data point of the standard curve) [33]. The limit of detection (LOD on column) was defined as the amount that could be detected with a signal-to-noise ratio of 3. The specificity of the assay for the analytes versus endogenous substances in the matrix was assessed by comparing the lowest concentration in the calibration curves with reconstitutions prepared with blank plasma from five different rats.

2.5.2. Accuracy and precision

The accuracy and precision (presented as the coefficient of variation; C.V.) of the assay were determined using QC samples. Accuracy (%) was determined from the percentage ratio of measured over nominal QC concentration (mean of measured/nominal×100). Intra-day precision was determined by analyzing replicate aliquots of QCs ($n=10$/each concentration) on the same day. Inter-day precision was determined by repetitive analysis of QC samples ($n=5$–10) on 3 consecutive days.

2.5.3. Recovery and ionization

Recovery was determined by comparing cleaned-up QC samples and cleaned-up drug-free plasma spiked with appropriate amount of standards. The efficacy of ionization was assessed by comparing spiked cleaned-up drug-free plasma to standard mixtures prepared directly in the reconstitution solvent.

2.5.4. Stability

The effect of different storage conditions on sample stability was determined for each analyte using QC samples. In one set of experiments, QC samples ($n=5$/each concentration) were kept for 6 h at room temperature before sample precipitation–reconstitution. In a different set of experiments, the stability of processed samples in the autosampler was determined using precipitated–reconstituted QC samples ($n=5$/each concentration) that were stored in capped 96-well plates for 24 h at 4 °C (temperature of the autosampler). Stability was assessed by comparing the mean concentration of the stored QC samples with the mean concentration of freshly prepared QC samples.

2.5.5. Application of the assay

The experimental protocol was approved by the Institutional Animal Care and Use Committee of AstraZeneca R&D Montréal. Rats were treated with a single iv bolus (10 μmol/kg) of morphine. Multiple blood samples were collected from the tail vein up to 4 h post-dose in test tubes containing EDTA. Following centrifugation (3000 *g*×10 min at 4 °C), plasma was collected and stored immediately at −80 °C until analysis.

3. Results and discussion

With the present method, following a simple protein precipitation from small volume samples (40 μl) and easy reconstitution steps, aliquots can be directly injected into the HPLC–MS system. The combined resolution and specificity of HPLC and MS allows maximum sensitivity from SIM analysis using only a single quadrupole. Since pharmacokinetic studies involve the analysis of numerous samples, the chosen method must combine accuracy and precision with speed and simplicity of execution.

3.1. Sample clean up and HPLC separation

In order to minimize sample preparation, our initial intention was to directly inject the diluted plasma samples after protein precipitation. Several solvents and diluted organic acids (e.g. 20% trifluoroacetic acid) were investigated, but only acetonitrile provided efficient protein precipitation that was compatible with MS. When sample composition contained more than 20% of organic solvent however, M and its metabolites were not adequately retained on analytical columns when MS compatible mobile phases were used. Consequently, the sample had to be evaporated and reconstituted in an aqueous

solution (0.1% formic acid) before injection into the HPLC–MS system. Using this simple reconstitution step, analyte recovery ranged from 70 to 93% (Table 1). M and NM recoveries were equal or greater than 89% at all concentrations tested while the glucuronide recoveries ranged from 70 to 78%. The lower recoveries observed may be explained by the polar nature of the glucuronide moiety or non-specific binding to certain components of the precipitated proteins.

Bioanalyses of complex mixtures consisting of small molecules often rely on HPLC–MS or MS–MS for the quantification of analytes. This can usually be achieved by targeting specific molecular masses or ion-transitions without extensive chromatographic separations [34]. However, since M3G and M6G possess identical mass to charge ratios and daughter ions corresponding to quasi-molecular ion of the parent compound, chromatographic resolution is a prerequisite. Consequently, a large variety of reversed-phase columns were investigated, including C_{18}, C_8, CN and phenyl–hexyl. The best separation was achieved with a phenyl–hexyl column using a step gradient of the mobile phase components. Complete separation was achieved within 8.0 min and analytes were detected at 3.9 (NM), 5.3 (M3G), 6.7 (M), 6.9 (M6G) and 7.5 min (I.S.). Analytes were identified on the basis of their retention times and mass spectra compared to individual standard solutions. Representative HPLC–MS chromatograms are presented in Figs. 2–4. More than 300 samples were injected on the column without adversely affecting its chromatographic performance. This was made possible by regularly washing the column and changing the pre-column 0.5-μm filter at the end of each day of analysis.

Table 1
Mean ionization efficiency and recovery of M and its metabolites

	Concentration (n*M*)	Ionization efficiency (%, mean, $n=5$)	Recovery (%, mean, $n=5$)
M	10	90	90
	400	94	91
	4000	98	90
NM	10	72	93
	400	71	91
	4000	86	89
M6G	10	113	78
	400	98	77
	4000	99	76
M3G	10	87	76
	400	94	70
	4000	97	70

3.2. Ionization and MS conditions

An important concern when applying AP-ESI–MS to quantitative bioanalyses is the ionization process within the ESI source, whereby the ionization efficiency relies on the vaporization of the mobile phase into fine droplets [35]. Factors such as high aqueous proportions, the presence of endogenous substances coming from the matrix or the possible co-elution of analytes can alter the process whereby the analytes are transferred from the mobile phase to the gaseous phase as ions [36,37]. Our experiment showed that post-column addition of acetonitrile improved the signal of NM and M3G. Since high proportions ($>$85%) of aqueous phase were present when NM and M3G were eluting, the high surface tension of the mobile phase was adversely affecting the desolvation process into the ion source and resulted in poor ion formation when compared to M or M6G [38,39]. Acetonitrile acted as a modifier, which reduced the radius of the droplet and enhanced ion formation.

Although chromatographic separation and post-column addition of acetonitrile were designed to maximize the ionization process, signal suppression could nevertheless occur since the MS detector only focused on selected ions (SIM mode). The results presented in Table 1 show that matrix suppression was minimal for M, M3G and M6G with a signal response always exceeding 85%. On the other hand, NM appeared to be more affected, as it displayed a maximal signal suppression of 30% in cleaned-up plasma samples compared to standard solutions.

3.3. Assay validation

Specificity was achieved by single quadrupole MS in SIM mode. Drug-free plasma showed no interfer-

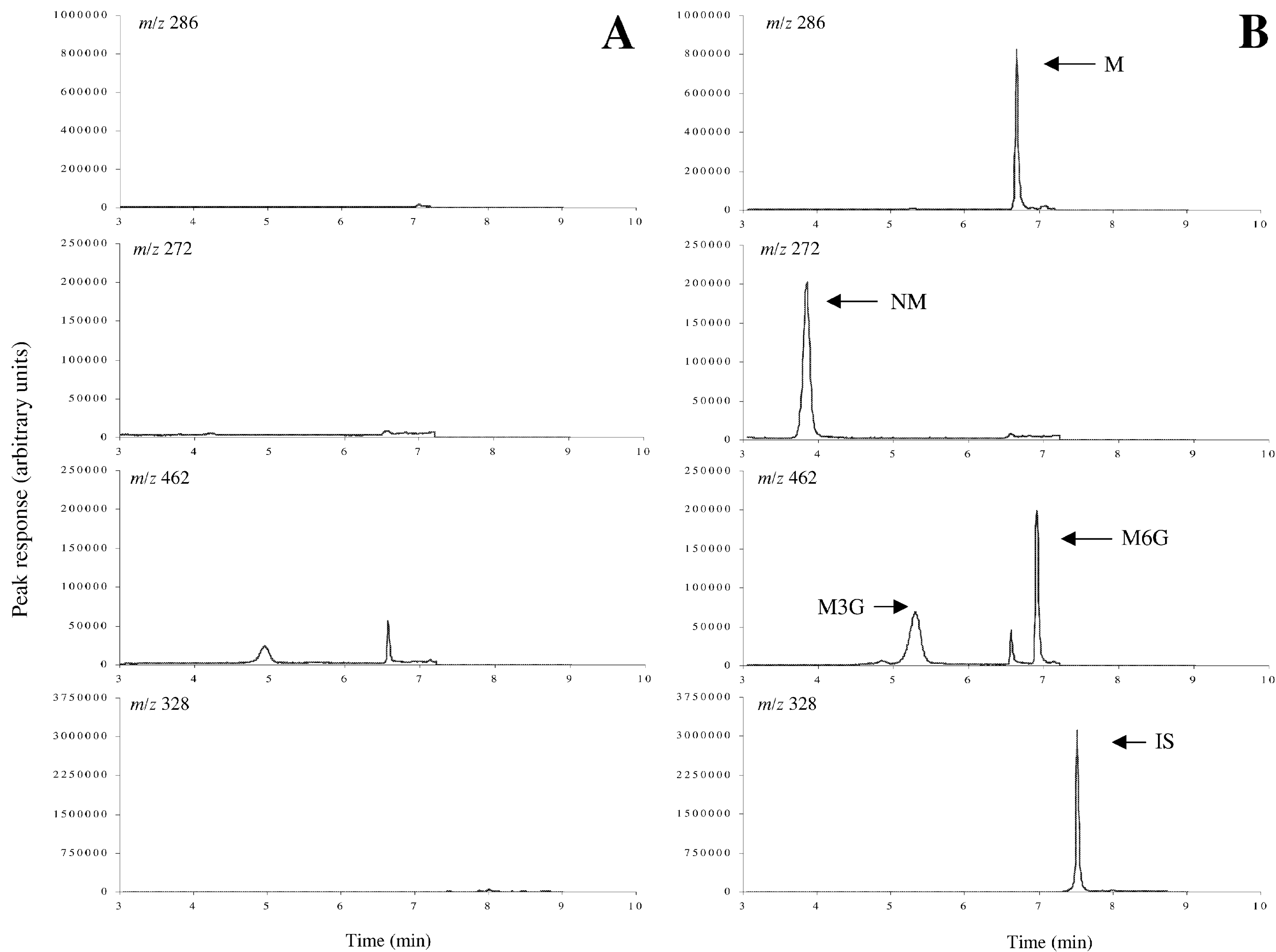

Fig. 2. LC–MS chromatograms of drug-free plasma (A), rat plasma spiked with 150 n*M* of M, NM, M3G and M6G (B). The quasi-molecular ions quantified were *m/z* 286 for M, *m/z* 272 for NM, *m/z* for M3G and M6G and *m/z* 328 for naloxone. Complete separation was achieved within 8.0 min and analytes were detected at 3.9 (NM), 5.3 (M3G), 6.7 (M), 6.9 (M6G) and 7.5 min (I.S.; naloxone).

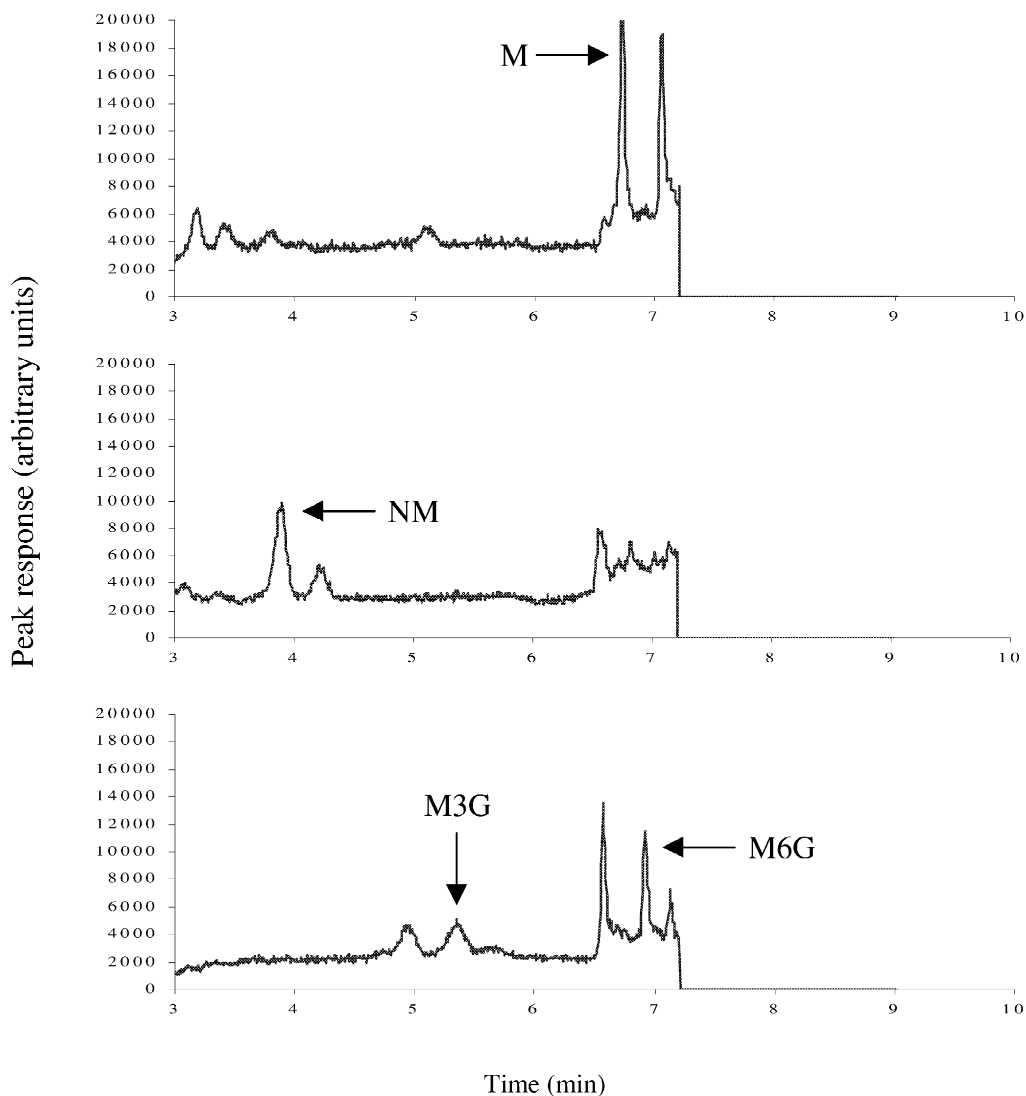

Fig. 3. LC–MS chromatograms of rat plasma spiked with LOQ concentrations of M (2.44 n*M*), NM (4.88 n*M*), M3G (4.88 n*M*) or M6G (4.88 n*M*).

ing peak with M, NM, M glucuronides or the I.S. at their respective retention times (Fig. 2A). To our knowledge, an HPLC–MS method with comparable sensitivity to HPLC–tandem MS has never been reported for quantitation of M and its metabolites. The sensitivity of the present assay was comparable or better than that reported in the literature from selected MS or MS–MS methods [26–32]. The absolute detection limits (signal-to-noise ratio =3) were 17, 10, 14 and 20 fmol for M, NM, M3G and M6G, respectively. The LOQ of the assay was 2.44 n*M* for M and 4.88 n*M* for its metabolites (Fig. 3).

The method allowed accurate measures over a wide range of concentrations (five orders of magnitude) and calibration curve determination coefficients (*r*-square) were better than 0.995. Residuals were always within 20% of the nominal value. The method was found to be accurate (91–98%) for M and NM and 85–97% accurate for M3G and M6G (Table 2). The intra- and inter-day precisions are

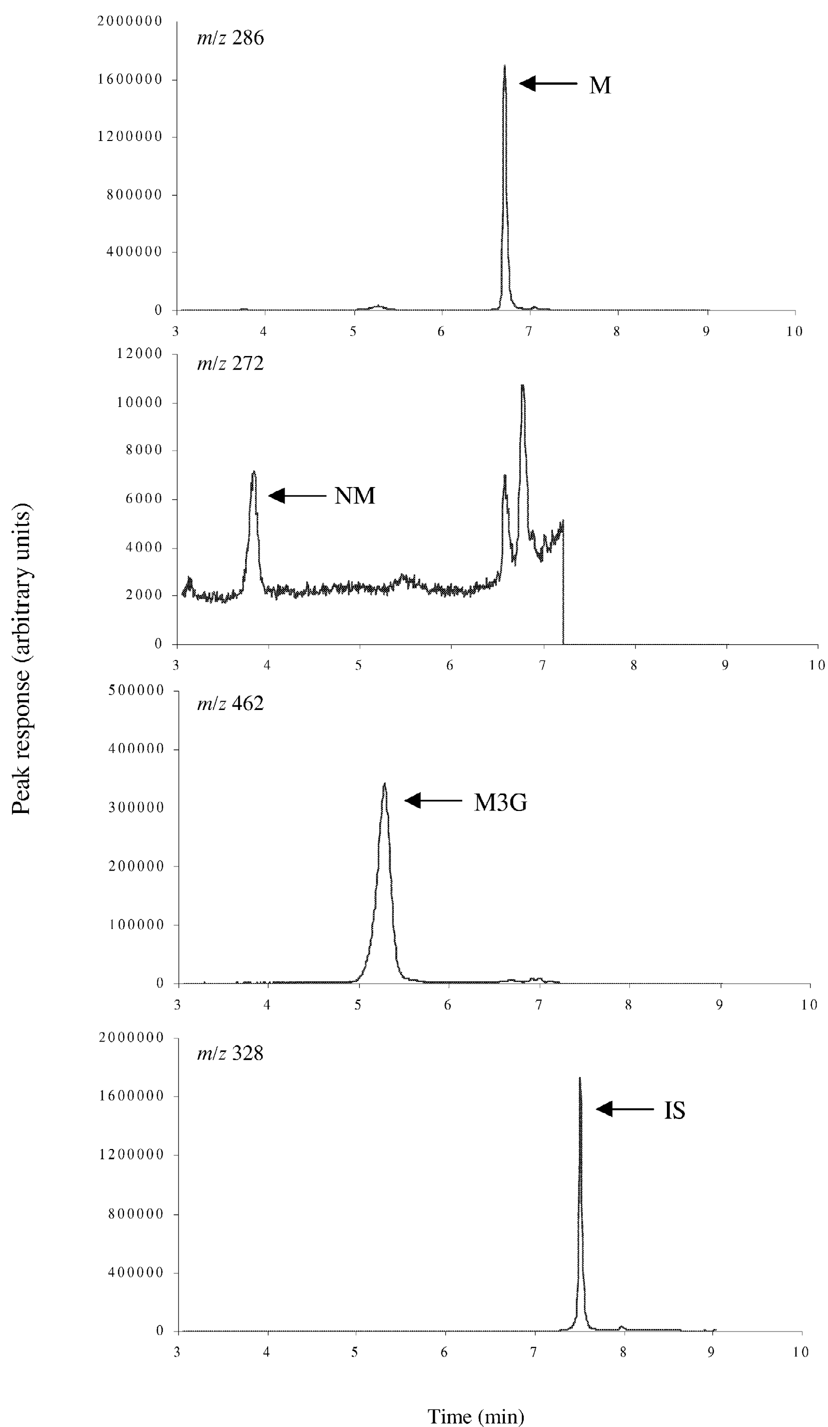

Fig. 4. LC–MS chromatograms of a rat plasma sample collected 1 h after an intravenous administration (10 μmol/kg) of morphine. Measured concentrations were 666 n*M* for M; 7.7 n*M* for NM and 1427 n*M* for M3G.

Table 2
Intra- and inter-day accuracy and precision data for M and its major metabolites

	Concentration (n*M*)	Intra-day (mean, $n=10$)		Inter-day (mean, $n=3$)	
		Accuracy (% nominal)	Precision (C.V., %)	Accuracy (% nominal)	Precision (C.V., %)
M	10	94	4.8	100	1.0
	400	96	1.9	92	3.9
	4000	91	3.1	87	5.0
NM	10	94	4.7	93	2.5
	400	95	1.4	93	0.1
	4000	97	3.3	94	2.9
M6G	10	86	6.0	94	7.0
	400	97	2.7	97	0.1
	4000	97	3.8	94	5.9
M3G	10	85	5.7	87	2.1
	400	95	3.0	95	3.3
	4000	98	5.9	95	5.0

presented in Table 2. Irrespective of the compound of interest or QC concentration, intra-assay precision (C.V.) was better than 6% and inter-day precision (C.V.), determined on three consecutive days, never exceeded 7%.

The effect of various storage conditions on sample stability is presented in Table 3. The experimental protocols were selected to allow enough time for sample preparation and overnight injections. The results revealed that reconstituted samples stored in the refrigerated autosampler and samples kept at room temperature for 6 h were not substantially

Table 3
Stability of M, NM, M6G and M3G after different storage conditions of QC samples

	Concentration (n*M*)	24 h stability (4 °C) (mean, $n=5$) (% control)	6 h stability (22 °C) (mean, $n=5$) (% control)
M	10	109	117
	400	103	105
	4000	101	101
NM	10	86	88
	400	93	97
	4000	95	97
M6G	10	118	82
	400	98	111
	4000	97	105
M3G	10	101	87
	400	98	108
	4000	95	101

C.V., coefficient of variation.

affected. For all analytes, calculated concentrations were within 20% of the value obtained from "untreated" QC standards.

3.4. Application of the assay

The method was used to study the PK of morphine and its metabolites in rats. Fig. 4 shows that M, M3G and NM were detected in plasma from one rat treated with a single iv bolus (10 μmol/kg) of morphine. The concentration versus time profile obtained over 4 h is comparable to those reported in the literature (Fig. 5) [32]. Micromolar concentrations of M were detected at 30 min and declined to approximately 100 n*M* at 4 h. The most abundant metabolite, M3G, surpassed levels of the parent compound after 30 min and its concentrations declined to approximately 1000 n*M* at 4 h. On the other hand, NM plasma concentrations never exceeded 30 n*M* and no detectable levels could be found after 1 h. This is in agreement with previous observation in morphine-treated rats where NM was found to be a minor metabolite in rat plasma and urine [4,32]. In agreement with data obtained in the literature, M6G could not be quantitatively measured [2,32].

4. Conclusion

In conclusion, a simple HPLC–MS method has been developed for the rapid and precise determination of M and its metabolites in small plasma samples (40 μl). Ninety-six samples could be prepared in a well-plate format within 3 h and a complete i.v. PK experiment (including calibration curves, QCs and samples obtained from four rats) could be analyzed in less than 24 h of injection time. From the very first sample collection to data processing, PK results could be provided to project teams in less than 3 days. Our results have demonstrated that HPLC–ESI–MS, together with a simple precipitation–reconstitution procedure, is an alternative to HPLC–MS or MS–MS methods that traditionally employed liquid–liquid or solid-phase extraction for the determination of M and its metabolites in biological fluids. The high sensitivity, rapid turnaround time and simplicity of the method make this technique particularly attractive for in vitro or in vivo PK studies.

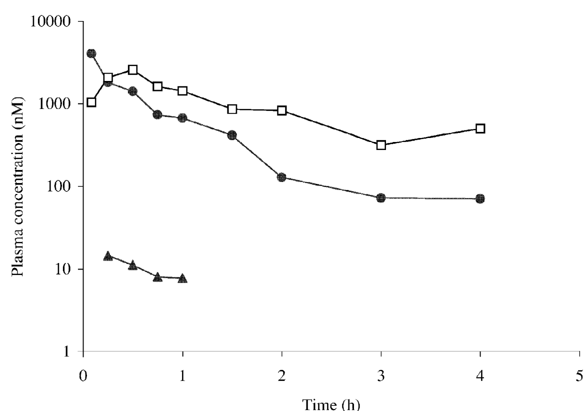

Fig. 5. Plasma concentration versus time profile of M (●), M3G (□) and NM (▲) after a 10-μmol/kg intravenous administration of morphine to one rat.

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