

Influence of Phenobarbital on Morphine Metabolism and Disposition: LC-MS/MS Determination of Morphine (M) and Morphine-3-Glucuronide (M3G) in Wistar-Kyoto Rat Serum, Bile, and Urine

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Abstract: A simple LC-MS/MS method has been developed and validated for the simultaneous determination of morphine (M) and morphine-3-glucuronide (M3G) in rat serum, bile, and urine. Deuterated D₃-M and D₃-M3G were used as internal standards (IS) for M and M3G, respectively. Serum samples were processed by acetonitrile precipitation. Bile samples were prepared by solid-phase extraction (SPE) using Oasis MCX cartridges. Urine samples were directly analyzed after dilution with mobile phase. Chromatography was performed using a Luna C₁₈ column (5 μm, 150 x 2.1 mm I.D.). The mobile phase consisted of acetonitrile (ACN) and 7.5 mM ammonium formate (pH 9.3) delivered from separate pumps with a simple gradient. The method was validated to quantify M in the range of 1-1000 ng/ml in bile and serum, and 0.025-25 μg/ml in urine. M3G was quantified in the range of 1-1000 ng/ml in serum, 0.1-100 μg/ml in bile, and 0.05-25 μg/ml in urine. The method was applied to study the pharmacokinetics and disposition of M and M3G in Wistar-Kyoto (WKY) rats, and the effect of phenobarbital (PB) on M and M3G disposition. M is metabolized to M3G at a lower rate in male than female rats leading to higher M levels and lower M3G levels in serum, urine, and bile of male than female rats. PB administration induces M glucuronidation to M3G in male, but not female WKY rats, and abolishes the gender differences in M and M3G pharmacokinetics.

Key Words: HPLC, mass spectrometry, morphine, morphine glucuronide, UGT, Wistar-Kyoto rats, phenobarbital, induction, CAR.

INTRODUCTION

Morphine (M) is the most widely used opioid analgesic for the treatment of chronic severe pain [1]. In humans, morphine is predominantly eliminated by glucuronidation. The major metabolites of morphine in humans are morphine-3-glucuronide (M3G), and to a lesser extent, morphine-6-glucuronide (M6G) [2-4]. Both metabolites are active, but with opposite pharmacological actions. M6G is a more potent analgesic than morphine [5], whereas M3G antagonizes both the pharmacological action and the respiratory depression side effects of morphine [6, 7]. However, in rats, M6G is formed in negligible amounts and therefore M3G is considered the only glucuronide metabolite of morphine [8-14].

Morphine exhibits a gender difference in its pharmacological action in humans [15], rats [16], and mice [17]. In rodents, morphine causes higher antinociception action in males than females [16, 17]. However inconsistent findings were reported in humans [15, 18]. It has been suggested that this gender difference in the antinociceptive action of morphine is due to differences in the pharmacokinetics of morphine, especially the rate of glucuronide formation [1]. Higher M3G:M ratios are detected in female than male rats [1, 19]. Furthermore, the hepatic UDP-glucuronosyltransferase that glucuronidates morphine (UGT2B1) is female pre-

dominant [20]. Therefore, because M3G is more rapidly excreted than M, and due to the antagonistic effect of M3G, the more rapid glucuronidation of M in female than males may explain why females are less sensitive to morphine than males.

Phenobarbital (PB) is an antiepileptic drug that is known to induce the expression of several metabolic enzymes, including UGTs [21]. PB induces the expression of its target genes *via* the activation of a transcription factor (nuclear receptor) known as constitutive androstane receptor (CAR) [22].

The Wistar-Kyoto (WKY) rat is a strain that has much higher expression of CAR in the liver of males than females [23]. Therefore, WKY is used as a model to test the role of CAR in induction and regulation of target genes expression [24]. If PB induces UGTs *via* CAR, then this inductive effect should be more prominent in male than female WKY rats. Consequently, the formation of the M3G metabolite in males and females should differ in response to PB. Therefore the WKY rat provides a model to investigate the molecular mechanism of the gender differences in the metabolism and disposition of morphine in untreated rats, as well as in response to PB treatment.

In order to understand the molecular basis of the gender differences in the pharmacokinetics and metabolism of morphine, a sensitive and valid analytical method is required to quantify morphine and its metabolites in various tissues and fluids. Several methods using various analytical techniques

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have been utilized for the quantitative analysis of morphine and its glucuronide metabolites in biological matrices. HPLC based methods coupled to UV [25-27], fluorescence [28-30], electrochemical [29, 31], coulometric [27]. Previous methods utilizing radioimmunoassays [32], gas chromatography (GC) [33], and capillary electrophoresis [34, 35] have also been used to quantify morphine and its metabolites.

LC-MS/MS analysis is becoming the analytical technique of choice for compounds in biological matrices because it provides high sensitivity, linearity, and selectivity [36]. Several LC-MS/MS were developed to quantify M and its glucuronated metabolites in human urine [37-39], human plasma [38, 40-45], human meconium [46] dog and monkey plasma [47], rat plasma [9, 48-50], and rat hair [9]. Most of these methods were designed to quantify morphine or morphine metabolites in plasma or urine with a limited dynamic range. There are no LC-MS/MS methods available in the literature for the quantification of morphine and its metabolites in bile. HPLC-UV or fluorescence methods were used to study biliary disposition of morphine [51, 52]. M and its glucuronated metabolites possess different physicochemical characteristics because of the high polarity of the glucuronide group. Therefore, extraction of both analytes with high efficiency from complex biological matrices such as bile, might be challenging and require multiple steps. However, because of the high sensitivity and selectivity of the MS methods, simple protein precipitation, and one step solid phase extraction were the most widely extraction techniques used to extract morphine and its metabolites from biological matrices.

The purpose of this study is to develop and validate a sensitive LC-MS/MS method for the simultaneous quantification of M and M3G in rat serum, urine, and bile. The assay presented in this paper is selective, has a low limit of quantification (1 ng/ml), large dynamic range (1000), and was validated with high precision, and accuracy. This analytical method was applied to a pharmacokinetic study to determine the molecular mechanism of gender differences in morphine antinociceptive actions as well as effects of PB on morphine metabolism in WKY male and female rats.

EXPERIMENTAL

Chemicals and Reagents

Morphine (M), morphine-3-glucuronide (M3G), and the deuterated internal standards D₃-morphine (D3M), and D₃-morphine 3-glucuronide (D₃-M3G) were obtained from Cerilliant (Round Rock, TX). HPLC-grade methanol, acetonitrile, and water were obtained from Fisher Scientific (St Louis, MO). Ammonium acetate, ammonium formate, ammonium hydroxide, formic acid, and acetic acid were obtained from Sigma-Aldrich (St Louis, MO). C₂ and C₁₈ solid-phase extraction (SPE) cartridges were obtained from Varian Inc (Palo Alto, CA). Oasis HLB and MCX SPE cartridges were purchased from Waters (Milford, MA).

Instrumentation

The HPLC system consisted of a Shimadzu SIL-20AC autosampler, a pair of LC-20AD pumps, a DGU-20A3 degasser, and a SCL-10AVP system controller (Shimadzu, Tokyo, Japan). The mass spectrometer was a Waters Quattro

Premier triple quadrupole instrument with an ESI source (Waters, Milford, MA). The entire LC-MS/MS system is controlled by MassLynx 4.0 software. All chromatographic separations were performed with a Phenomenex Luna C₁₈ column (5 μm, 150 x 2.1 mm I.D.) equipped with a Luna C₁₈ guard column (Phenomenex, Torrance, CA).

Liquid Chromatographic and Mass Spectrometric Conditions

The Luna C₁₈ column was used for all chromatographic separations. The mobile phase consisted of ACN (mobile phase A) and 7.5 mM ammonium formate adjusted to pH 9.3 using 10 M ammonium hydroxide and formic acid (mobile phase B). The two mobile phases were delivered from 2 separate LC pumps. The gradient profiles for the LC pumps are shown in Table 1. The LC flow was split after the column directing half the flow toward the MS instrument and the other half to waste. A built-in switching valve was used to direct the LC flow to the MS instrument from 2-6 mins during each run and to waste for the rest of the run time and between runs. The injection volume of all samples from the 3 biological matrices was 10 μl.

Table 1. HPLC Gradient Profiles for M and M3G in Rat Bile, Serum, and Urine. %A is the Percentage of Mobile Phase A (ACN) in the Total LC Flow

Bile		Serum and Urine		Flow (ml/min)
Time (min)	% A	Time (min)	% A	
0:00	10	0:00	10	0.7
2:00	10	2:00	10	0.7
4:00	40	4:00	40	0.7
5:15	40	5:15	40	0.7
5:30	90	5:30	50	0.9
6:30	90	6:00	50	0.9
6:45	10	6:15	10	0.9
8:45	10	7:30	10	0.9

The mass spectrometer was set to the positive ion mode of operation, with the source and desolvation temperatures of 125, and 400°C, respectively. The capillary, cone, and extractor voltages were 3000 V, 45 V, and 6 V, respectively. The source and desolvation gases (Nitrogen) were set at a flow rate of 750 and 100 L/h, respectively. The collision energies were 25 eV for M and D₃-M, 30 eV for D₃-M3G. The collision energy for M₃G was set at different levels for each matrix (30 eV for serum, 20 eV for urine, and 16 eV for bile). The multiple reaction monitoring (MRM) transitions were 462/286, 465/289, 286/201, and 289/201 for M3G, D₃-M3G, M, and D₃-M, respectively. Each transition was assigned a dwell time of 50 milliseconds. Fig. (1) shows the chemical structures of M and M3G.

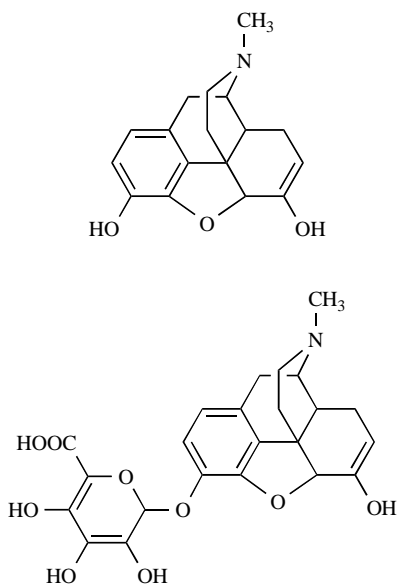


Fig. (1). Chemical Structures of morphine and morphine-3-glucuronide.

Ionization Suppression and Mobile Phase Effect on MS Signal

The ionization suppression regions for the different matrices were determined using post-column infusion of a 1 $\mu\text{g/ml}$ mixture of M and M3G from an infusion pump at a rate of 10 $\mu\text{l/min}$ according to the procedure of King and co-workers [53]. Briefly, a 0.7 ml/min HPLC make-up flow of 10% mobile phase A (ACN) and 90% mobile phase B (7.5 mM ammonium formate) were combined, using a zero dead volume T junction, with 1 $\mu\text{g/ml}$ mixture of M and M3G delivered from a separate infusion pump at a flow rate of 10 $\mu\text{l/min}$. Blank samples from the serum, bile, and urine were extracted and 10 μl was injected. The suppressive effect of the matrix endogenous components was evaluated qualitatively by monitoring the depression in MS signal of M and M3G.

The same technique was used to study the effect of various aqueous mobile phases on the intensity of the MS signal. Every 2 mins, the mobile phase stock bottle was replaced with a different bottle containing new mobile phase, and the pumps were purged with the new mobile phase for 1 min before the LC make-up flow and the analyte infusion pump started delivering again.

Preparation of Standard Solutions and Calibration Curves

One mg/ml stock solutions of M, M3G, D₃-M, and D₃-M3G were individually prepared in water: ACN (1:1). Blank serum, bile, and urine were collected from untreated animals. A 100 μl of blank serum was spiked with 50 μl of the appropriate standard solution and 50 μl of the IS solution to construct a calibration curve with the range of 1-1000 ng/ml for both M and M3G. Individual calibration curve points were of concentrations: 1, 2, 5, 10, 100, 500, and 1000 ng/ml. The concentration of both D₃-M and D₃-M3G internal standards was 100 ng/ml for both. 100 μl of bile was spiked with 50 μl

of the appropriate standard solution and 50 μl IS solution to construct a calibration curve of 1-1000 ng/ml for M and 0.1-100 $\mu\text{g/ml}$ for M3G. Individual calibration curve points were of concentrations: 1, 2, 5, 10, 100, 500, and 1000 ng/ml for morphine and 0.1, 0.2, 0.5, 1, 10, 50, and 100 $\mu\text{g/ml}$ for M3G. The concentration of the D₃-M and the D₃-M3G internal standards were 100 ng/ml and 1 $\mu\text{g/ml}$, respectively. 50 μl of blank urine was spiked with 50 μl of the appropriate standard solution and μl of IS solution to construct a calibration curve of 0.025-25 $\mu\text{g/ml}$ for both M and M3G with individual calibration points of concentrations 0.025, 0.05, 1, 0.25, 2.5, 12.5, and 25 $\mu\text{g/ml}$. The concentration of both D₃-M and D₃-M3G internal standards was 1 $\mu\text{g/ml}$.

Sample Extraction

Several solid-phase extraction and protein-precipitation techniques were investigated for sample clean-up. Solid-phase extraction (SPE) using C₁₈, C₂, Oasis-HLB, and Oasis-MCX cartridges were investigated. For serum samples, simple protein precipitation using ice-cold acetonitrile was used. 500 μl of iced-cold acetonitrile was added to 100 μl serum samples spiked with 50 μl IS, vortexed, and centrifuged at 12,000 rpm for 10 min. The supernatant was aspirated, evaporated under vacuum, and reconstituted in 200 μl of 10% ACN.

For bile samples, Oasis-MCX SPE cartridges resulted in the highest extraction recovery of all clean-up techniques that were investigated. The spiked bile samples were loaded onto SPE cartridges, washed with 2 ml of 2% HCl followed by 2 ml methanol, and eluted with 1 ml of 5% NH₄OH in 60% methanol. The eluate was then evaporated under vacuum and reconstituted in 200 μl of 10% ACN. Urine samples (50 μl) were spiked with IS and diluted 20 fold by the addition of 900 μl of 10% ACN (1 ml final volume), vortexed, and directly used for LC-MS/MS analysis.

Extraction recoveries were determined for each QC point in each matrix. To determine the extraction recovery, five blank samples of each QC point in each matrix were extracted and then spiked with the appropriate analyte standard solutions. The same number of blank samples was spiked with the same analytes standard solution prior to extraction. Additionally, neat samples were prepared by spiking neat solution (mobile phase solvent) with the same analyte standards.

Absolute extraction recovery was calculated as the ratio of the analyte peak area in samples spiked before extraction compared to the corresponding peak area in untreated samples prepared in neat solution (total). Relative extraction recovery was calculated as the ratio of analyte peak areas in samples spiked after extraction compared to the corresponding peak area in untreated samples prepared in neat solution (suppression).

Method Validation

The method was validated using 5 quality control (QC) points for each calibration curve. Five replicates of each QC point were analyzed each day to determine the intra- and inter-day accuracy and precision. This process was repeated 3 times over 3 days in order to determine the inter-day accu-

racy and precision using freshly prepared calibration curves. Intra-day accuracy and precision were calculated from the % bias [% (Measured – Theoretical) / Measured concentrations] and %RSD [% Standard Deviation/ Mean], respectively, for the 5 replicates of each QC point. Inter-day accuracy and precision were calculated the same way for the 15 replicates of each QC point pooled from the 3 validation runs. The concentrations of the QC points for serum were 1, 3, 50, 750, and 1000 ng/ml for both M and M3G. Bile QC points were 1, 3, 50, 750, and 1000 ng/ml for M and 0.1, 0.3, 5, 75, and 100 µg/ml for M3G. The concentrations of urine QC points were 0.025, 0.075, 1.25, 18.75, and 25 µl for both M and M3G.

Method selectivity was evaluated by injecting blank-extracted samples from the three matrices and monitoring the presence of any peaks with similar elution time to both analytes and the internal standard. Carry over was evaluated by injecting samples of the highest concentrations in a calibration curves followed by blank samples and monitoring the peak area of any any peaks with similar elution time to both analytes and the internal standard.

Animal Studies

Male and female WKY rats were purchased from Taconic Farms Inc. (Hudson, NY), were 8-10 weeks old, and weighed 210-370 g. Animals were housed in a temperature-, light-, and humidity-controlled environment according to the American Animal Association of Laboratory Animal Care guidelines. Five male and 5 female rats were used per treatment group. The rats were fed Laboratories Rodent Chow W (Harlan Laboratories, Madison, WI) and water *ad libitum*. Male and female WKY rats were pretreated for four days with saline (5 ml/kg) or phenobarbital (80 mg/kg). Approximately 24 hrs after the last dose, rats were placed in metabolic cages following subcutaneous administration of morphine (7.5 mg/kg). Rats were placed under isoflurane anesthesia and approximately 250 µl blood samples were collected from the orbital sinus into Microtainer serum separator tubes (Fisher Scientific) at 0.5, 1, 2, 4, 6, and 22 hr from each animal after dosing with morphine. Antiseptic was placed on the eye after each bleed and the rats were returned to the metabolic cages. Urine was collected from metabolic cages at 4, 10, and 22 hr from each animal following administration of morphine. Following a three-day washout period, rats were retreated as mentioned previously. Approximately 24 hrs after the last pretreatment, rats were placed under anesthesia consisting of 75 mg/kg ketamine and 10 mg/kg xylazine, and a canula was placed in the bile duct. Animals were then dosed with morphine (2.2 mg/kg) as an iv bolus. Bile was collected from the canula at 20, 40, 60, 90, and 120 min.

Pharmacokinetic Analysis

M and M3G serum data were analyzed by non-compartmental using WinNonlin (Pharsight, Mountain View, CA, USA) to calculate AUC. Cumulative amount excreted in bile and urine was calculated by adding the amount measured in each data point of the excretion profile to the amount measured in the earlier data points.

RESULTS AND DISCUSSION

Development of Chromatographic and Mass Spectrometric Conditions

Structures of morphine and M3G are shown in Fig. (1). Morphine is a weak base (pKa = 7.9) [54]. Therefore, increasing the pH of the mobile phase results in increasing the retention time of both analytes. However, the glucuronide group in M3G is always present in the ionized form in the pH range commonly used for reverse-phase chromatography (pH 3-10) [55]. Therefore, changing the pH of the mobile phase increases the elution time of morphine more than that of M3G. Fig. (2) demonstrates the effect of the pH of the aqueous component of mobile phase with 10% ACN on the elution time of M and M3G.

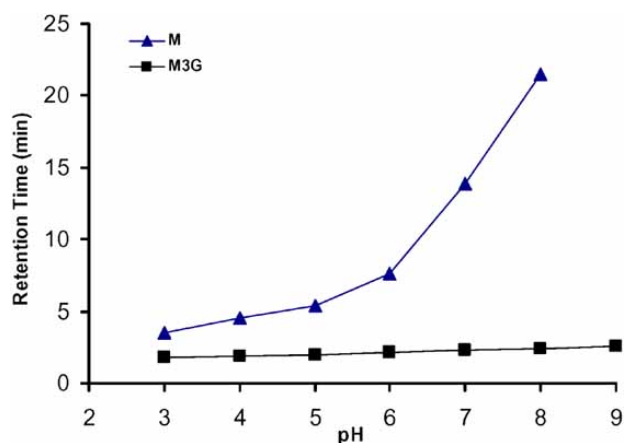


Fig. (2). The effect of the pH of the mobile phase on the elution time of M and M3G. The mobile phase consists of 10% ACN in 7.5 mM ammonium formate at a flow rate of 0.7 ml/min.

The effect of mobile-phase pH on the MS-signal sensitivity was also investigated to maintain the balance between optimum chromatographic and MS conditions. The positive-ionization mode was used for the MS detection of both M and M3G. Therefore, we expected the high-pH mobile-phase conditions, favored for chromatography, to suppress the formation of positively charged ions and consequently the MS signal. Surprisingly, the MS signal increased for both M and M3G with increased pH. Fig. (3) shows the influence of pH on the MS signal of M3G. Increasing the mobile-phase pH probably provided better conditions for droplet formation and dissolution, however it cannot be explained by increasing ion formation in the solution phase.

A mobile-phase pH higher than 10 was avoided to ensure a longer half-life of the column and seals of the HPLC system. Therefore, 0.1% NH₄OH (pH>10) was not considered as a mobile phase of choice. Ammonium formate (7.5 mM, pH 9.3), at flow rate 0.7 ml/min, was found to provide optimum peak shape, retention time, and MS signal. Under these conditions, elution time for M was more than 20 min. Therefore, a steep gradient increasing the organic component (ACN) of the mobile phase from 10 to 40% was selected to elute M faster. A steeper gradient resulted in high ion-suppression effects of the serum, bile, and urine matrices due

to co-elution of unknown endogenous components of the biological matrices with the analytes of interest. Furthermore, washing the column with 90% ACN (1 min) for bile samples, and 50% ACN (30 sec) for urine and serum samples was required to eliminate the cumulative suppressive effect resulting from repetitive injections. The flow was split to direct half to waste to ensure complete evaporation of the capillary spray in the MS source. Fig. (4) shows a representative chromatogram of M and M3G in bile under the final chromatographic conditions.

The MS instrument was tuned to obtain the highest MS signal for analytes and internal standards. The MS signal (ion count) for M3G was very high at the upper part of the bile (0.1-100 µg/ml) and urine (0.025-25 µg/ml). The high ion count of M3G decreased the linearity of M3G calibration curve in these matrices and caused the method to fail validation. Urine and bile samples contained lower levels of M than M3G, therefore they were not further diluted. Instead, the MS signal intensity of M3G in bile and urine was reduced without affecting the M signal intensity, by using

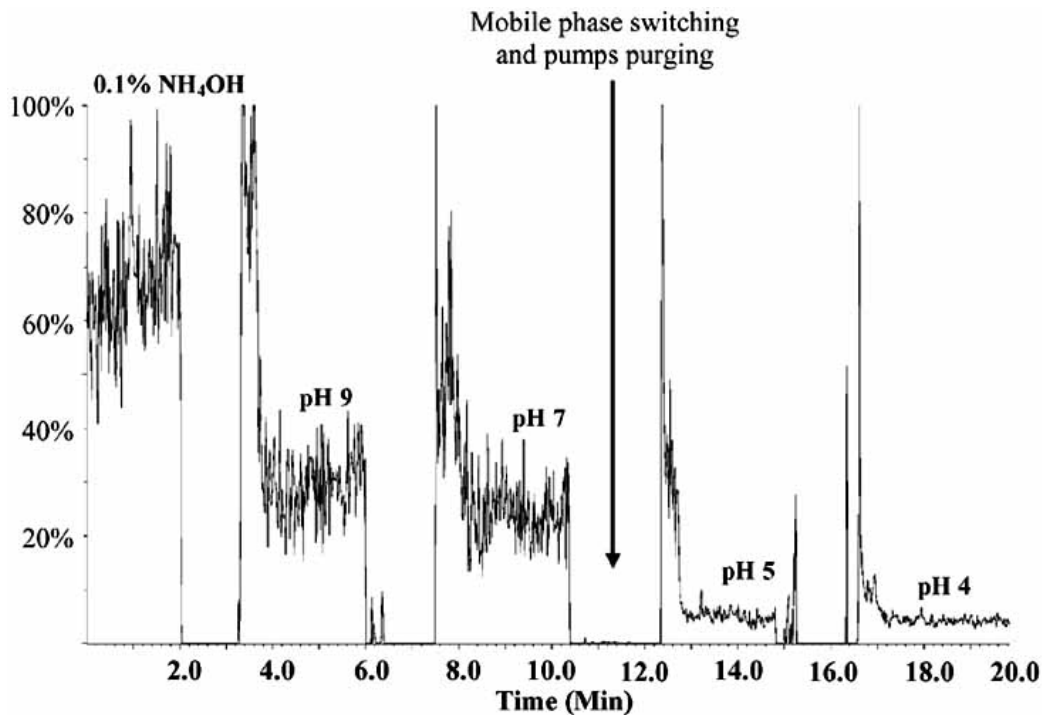


Fig. (3). The effect of mobile phase (7.5 mM Ammonium formate) pH, adjusted with formic acid, on MS signal intensity of M3G.

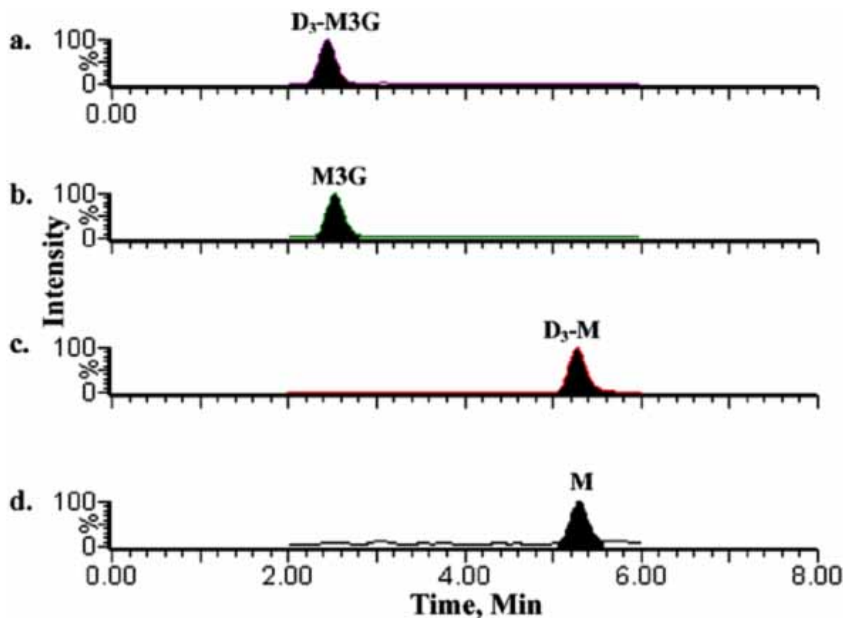


Fig. (4). Representative chromatograms of (a) D₃-M3G, (b) M3G, (c) D₃-M, and (d) M in serum at QC1 level. Concentration of the analytes (M and M3G) is 1 ng/ml and concentration of deuterated internal standards (D₃-M and D₃-M3G) is 100 ng/ml.

lower collision energy for M3G (20 ev in urine, 16 ev in bile). Under these conditions, M3G produced linear calibration curve under the range of (0.1-100 µg/ml) for bile, and (0.025-25 µg/ml) for urine, without further diluting the samples, which would decrease the limits of detection for M. Therefore, acceptable signal intensities in the range for which this assay was developed was achieved for both M and M3G. Signal/noise (S/N) ratios as measured by peak height/Peak height, of the lowest point in the calibration curve were 32 in serum, 500 in urine, and 340 in bile. For M3 S/N ratios were 26 in plasma, 240 in urine, and 18 in bile.

Development of Sample Extraction Conditions

Several protein-precipitation and SPE methods were investigated to increase extraction recovery and decrease suppression effect of the matrix. The large difference in polarity between M and M3G caused different extraction efficiencies of the two analytes. Because this method was developed for the simultaneous quantification of M and M3G, extraction conditions were optimized to ensure the highest extraction efficiency possible of both analytes. Furthermore, the high sensitivity of the MS detector allowed for lower limits of detection than required for this application.

To help develop sample extraction conditions, early on in method development, the ion-suppression effect was evaluated by making an injection of a blank biological sample after extraction with various conditions and under the final chromatographic conditions. The MS-signal intensity was monitored from a continuous infusion of the analytes combined with the LC effluent coming from the column. Ideally, the resulting chromatogram should not contain ion-suppression areas in the time window, where any of the analytes elute. Extraction procedures that cause ion-suppression throughout the run are early excluded from method devel-

opment. Fig. (5) shows an example of the matrix effect of bile extracted with acetonitrile protein precipitation. In this case, it is clear that the matrix effect exists throughout the entire period of the run, and will suppress the analytes signal regardless of their retention time. Therefore, protein precipitation using acetonitrile was early excluded in method development to extract bile samples. Fig. (6) shows the matrix effect of serum, urine, and bile after preparation under final extraction conditions. It is clear that suppressive-effect of the matrix does not exist in the time window, where analytes elution take place.

Quantitative evaluation of the extraction efficiency was determined by calculating the absolute and relative extraction recoveries. Absolute recovery of the analyte from the matrix depends on extraction efficiency, suppression, and analyte binding to matrix. Relative extraction recovery determines the signal suppression resulting from the endogenous components of the matrix co-extracted with the analyte of interest. Therefore, relative recovery can be improved by either adjusting the extraction conditions to prevent the co-extraction of certain interferences, or adjust the chromatographic conditions to separate the interferences from the analyte peak. When relative recovery is improved and yet absolute recovery is still low, this indicates significant loss of analyte during precipitation, phase separation, washing, or elution that can be improved by adjusting the extraction conditions.

Table 2 demonstrates the absolute and relative extraction recoveries of bile samples prepared using several solid-phase extraction (SPE) and protein-precipitation (PP) extraction methods. PP extraction methods yielded very poor absolute and relative recoveries for both M and M3G. The low-relative recovery indicates the severe ion suppression resulting from bile matrix. This matrix effect was not improved by using different chromatographic conditions, because signal

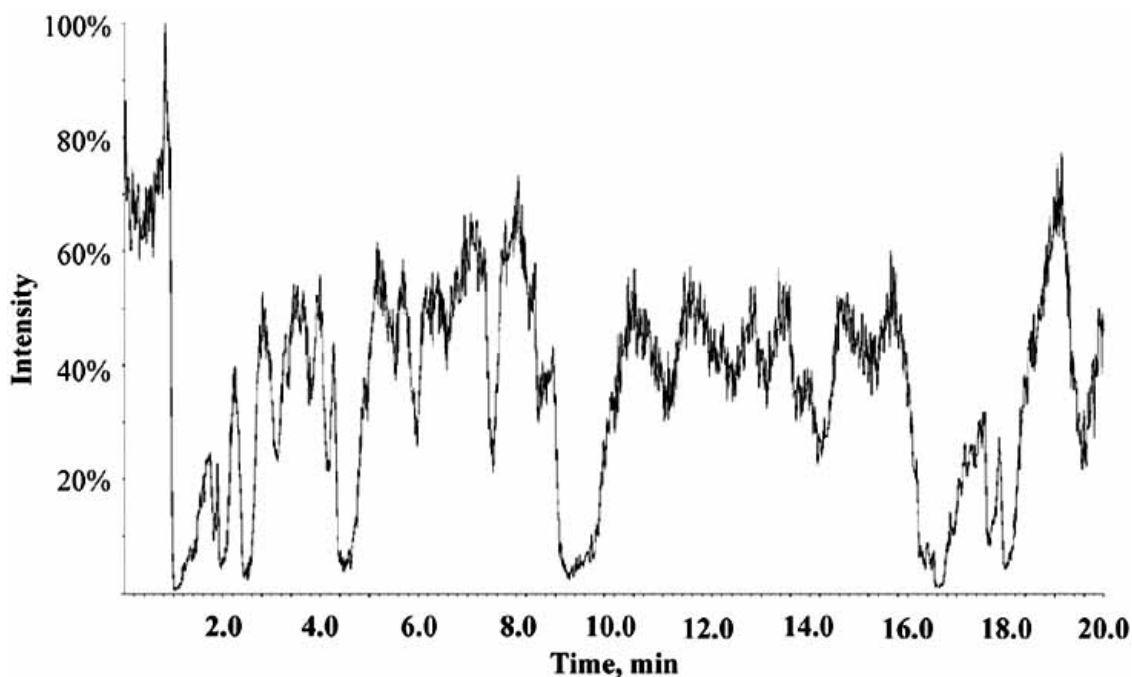


Fig. (5). The suppressive effect of a blank bile sample extracted by acetonitrile precipitation on the M3G MS signal.

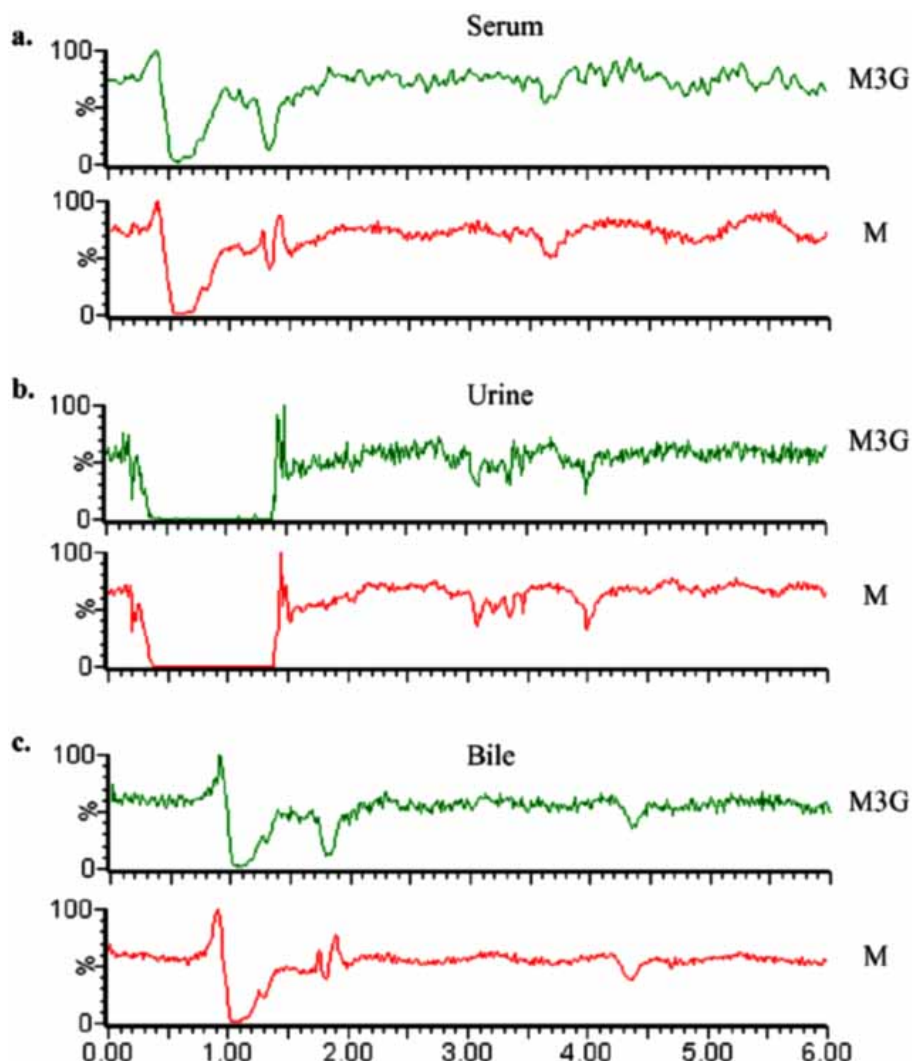


Fig. (6). The suppressive effect of a) serum, b) urine, and d) bile after preparation under final extraction conditions on M and M3G MS signals.

suppression was not restricted to a specific period of time during the run, but was spread throughout the entire chromatogram (Fig. 5). Therefore, early in method development, PP was excluded as a method of choice for extracting bile samples.

Different SPE extraction cartridges with different conditioning, washing, and elution conditions were investigated to obtain acceptable extraction recoveries for M and M3G in bile samples. C₂, C₁₈, and Oasis-HLB cartridges yielded better extraction recoveries than PP methods, however severe matrix effects still existed. Oasis-MCX cartridges yielded the highest absolute extraction recovery, and most importantly, resulted in a minimum matrix effect. Matrix effect is generally inconsistent, and is considered the most common reason for a method to fail validation. Contrary to the matrix effect, absolute recovery is generally more consistent, and low-absolute recovery does not necessarily cause method failure, especially in the presence of a proper internal standard. Consequently, Oasis-MCX cartridges were selected for extraction of bile samples in this study, despite the low absolute recovery, because it yielded high relative recoveries.

Table 2. Relative and Absolute Extraction Recoveries of 100 ng/ml M and M3G in Bile Using Different Solid-Phase Extraction (SPE) Cartridges and Protein Precipitation (PP) Methods

Method	M		M3G	
	Relative	Absolute	Relative	Absolute
C ₂ SPE	38%	12%	28%	8%
C ₁₈ SPE	23%	16%	16%	8%
Oasis HLB SPE	42%	28%	36%	11%
Oasis MCX	88%	35%	94%	33%
ACN PP	15%	8%	5%	< 5%
Acid PP	13%	7%	4%	< 5%
Base PP	12%	8%	4%	< 5%

Serum and urine matrices are much cleaner than bile and did not require as elaborate optimization of sample preparation conditions. Simple dilution of urine samples with mobile phase and ACN-protein precipitation of serum samples resulted in high extraction recovery. Absolute extraction recovery of serum, urine, and bile QC points under the final extraction conditions are listed in Table 3.

Method Validation

Assay precision and accuracy were calculated for each matrix over a 3-day interval. Intra-day (n=5) precision and

accuracy were calculated from the quantification of 5 samples at each QC point on 3 separate days (data not shown). Five QC concentrations, including the lowest concentration, 3 times the lowest concentration, a concentration in the lower half, 75% of the highest concentration, and the highest concentration of the calibration curve was used for each matrix. Inter-day precision, as expressed by %RSD, and accuracy as expressed by % bias for M and M3G in the 3 biological matrices, are shown in Table 4. Inter-day precision and accuracy for M ranged from 6.9-13.7 % and minus 15.4-8.7 % respectively; whereas for M3G it ranged from 5.2-

Table 3. Absolute Extraction Recoveries \pm RSD of the 5 QC Levels Used to Validate the Calibration Curves of M and M3G in Serum, Urine, and Bile. The Concentrations of M and M3G QC Points in the Different Matrices are Listed in the Experimental Section. Data were Pooled from the 15 Samples Ran Over the 3-Validation Days for Each QC Point

QC	Serum		Urine		Bile	
	M	M3G	M	M3G	M	M3G
1	89 \pm 10%	70 \pm 11%	89 \pm 9%	91 \pm 11%	47 \pm 7%	33 \pm 13%
2	86 \pm 9%	72 \pm 10%	90 \pm 6%	86 \pm 8%	44 \pm 10%	31 \pm 9%
3	83 \pm 5%	69 \pm 8%	90 \pm 3%	78 \pm 7%	43 \pm 3%	30 \pm 10%
4	85 \pm 4%	75 \pm 7%	98 \pm 6%	80 \pm 8%	43 \pm 6%	29 \pm 7%
5	86 \pm 8%	69 \pm 8%	96 \pm 6%	87 \pm 6%	36 \pm 10%	32 \pm 9%

Table 4. Interday Precision (% RSD) and Accuracy (% Error) for M and M3G in Serum, Urine, and Bile (N=15). Concentrations in Serum are in ng/ml, in Urine in μ g/ml, and in Bile in ng/ml for M and μ g/ml for M3G

Theoretical Conc		Measured Conc		% RSD		% Bias	
M	M3G	M	M3G	M	M3G	M	M3G
Serum							
1	1	1.000	0.961	13.7	14.1	0.1	-3.9
3	3	3.014	3.275	10.6	5.2	0.45	9.2
50	50	51.68	48.25	8.4	11.9	3.4	-3.5
750	750	746.8	802.6	8.4	7.8	-0.6	7.0
1000	1000	992.8	1002.5	9.1	13.2	-0.7	0.3
Urine							
0.025	0.025	0.021	0.023	14.2	10.6	-15.4	-8.9
0.075	0.075	0.078	0.070	10.4	8.7	3.9	-6.8
1.25	1.25	1.241	1.259	12.6	10.4	-0.7	0.7
18.75	18.75	17.80	17.88	9.9	10.0	-5.1	-4.6
25	25	25.50	24.82	9.5	9.2	2.4	-1.3
Bile							
1	0.1	0.988	0.094	12.2	14.2	-1.2	-6.1
3	0.3	3.001	0.312	9.1	9.8	0.1	4.2
50	5	47.88	4.680	10.4	9.5	0.7	-6.4
750	75	815.4	69.37	6.9	7.5	8.7	-7.5
1000	100	955.1	107.0	9.8	7.1	-4.5	7.0

14.1 % and minus 8.9-9.2 %, respectively. Individual intra-day accuracy and precision for each QC point in each matrix were less than 15% (or 20% for LLOQ) (data not shown). All calibration curves were weighted according to the 1/x²-weighting scheme. The calibration curves showed acceptable linearity (R² > 0.98) for M and M3G in serum, urine, and bile. No significant interfering peaks from injecting any of the blank matrices were detected, which demonstrates method selectivity. Blank samples injected after the injection samples with the highest concentration did not yield any significant peaks with peak area highest than 10% of the peak area of the lowest concentration (LLOQ) in each calibration curve. Therefore, potential problems caused by carryover were not encountered.

Animal Studies

This analytical method was applied to examine the gender difference in serum, bile, and urine pharmacokinetics of morphine and its 3-glucuronide metabolite in WKY rats. The gender difference in the effect of PB pretreatment on morphine and its metabolite was also examined. Figs. (7), (8), and (9) show a representative serum concentration, cumulative amount excreted into bile, and cumulative amount excreted into urine, vs. time profiles, respectively.

Table 5 summarizes serum, bile, and urine pharmacokinetics of M and M3G in the different treatment groups. In control animals, serum morphine concentrations are lower in female than male rats, whereas M3G is higher in female than male rats. This gender difference might be a result of the higher levels of UGT2B1 (the main UGT isoform responsible for morphine glucuronidation in rats [20]) in female relative to male rats. The higher M levels in male than female rats is thought to be the reason male rats are more sensitive to the antinociceptive action of morphine [19].

Table 5. Summary of M and M3G A) AUC in Serum (µg.min/ml), B) Cumulative Amount Excreted in Bile Over 2 Hours (ng for M, and µg for M3G), and C) Cumulative Amount Excreted in Urine Over 22 Hours (µg)

Male Female	Saline	PB	Saline	PB
A) Serum AUC				
M	94.6	52.0	79.1	74.6
M3G	65.41	124.1	129.1	121.7
B) Cumulative Amount Excreted in Bile				
M	2719	1652	1475	1504
M3G	166	366	308	330
C) Cumulative Amount Excreted in Urine				
M	318	199	191	180
M3G	112	276	354	313

Treating the male WKY rats with PB increased the M3G serum concentrations markedly, and decreased the morphine serum levels to a lesser extent, bringing both M and M3G to

similar concentrations as in females. However, both morphine and M3G levels in female WKY rats are not affected by PB. Therefore, PB treatment eliminated the gender differences in the M:M3G serum concentration ratios. Data from our laboratory demonstrated that PB administration abolishes the gender difference in the antinociception action of morphine (manuscript in preparation). Also, other data from our laboratory demonstrates that PB induces the expression of UGT2B1 in male WKY rats only (manuscript in preparation). The gender specific effect of PB on the pharmacokinetics of M and M3G might explained by the gender difference in CAR expression. Because UGTs induction by PB is mediated *via* the nuclear receptor CAR, and because female WKY rats have low expression of CAR, PB does not induce

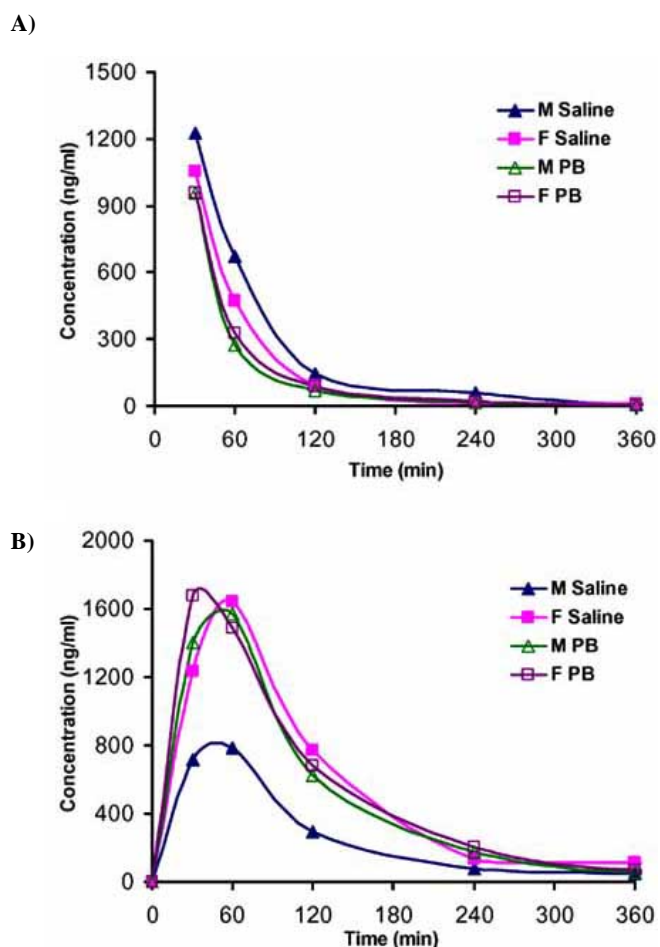
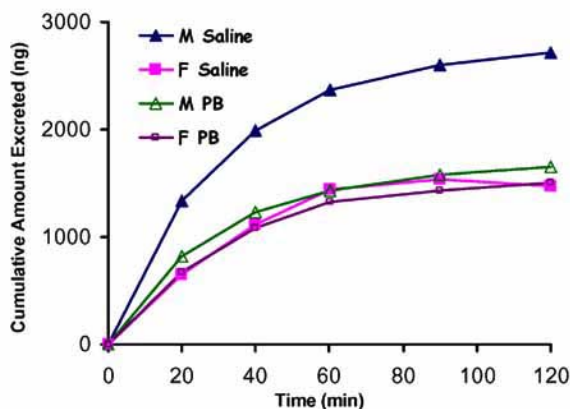


Fig. (7). Concentration vs. time serum profiles of (A) M and (B) M3G in the 4 treatment groups after subcutaneous administration of M.

UGT expression, and consequently does not affect morphine pharmacokinetics in female WKY rats. M and M3G levels in bile and urine reflect the serum pharmacokinetics. Changes in M and M3G serum levels among the different treatment groups were associated with similar changes in bile and urine, that is an increase in M or M3G serum concentrations was accompanied with an increase in bile and urine concen-

trations maintaining a constant ratio of urine/serum and bile/serum concentrations of M and M3G. Therefore, changes in bile and urine concentrations of M and M3G resulted from their changes in serum concentrations, and did not indicate any effect on transport kinetics between serum and urine, or serum and bile.

A)



B)

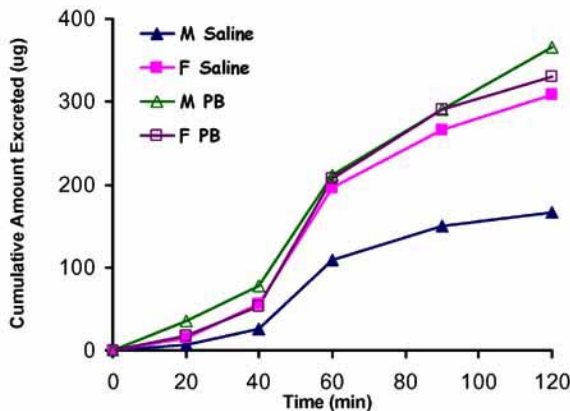


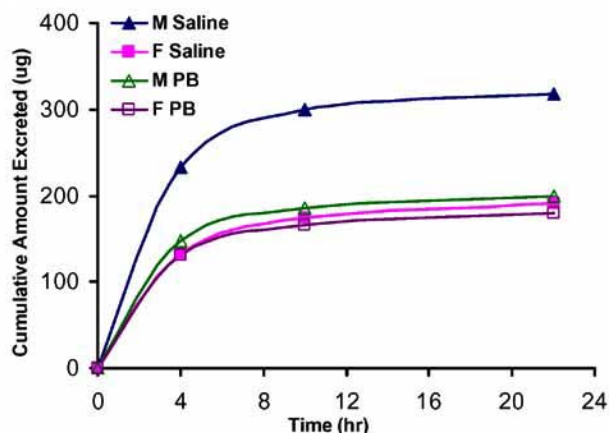
Fig. (8). Cumulative amount excreted in bile vs. time profiles of (A) M and (B) M3G in the 4 treatment groups after I.V. administration of M.

CONCLUSION

A sensitive, efficient, and accurate method was developed and validated for the simultaneous quantification of M and M3G in rat serum, urine, and bile. Solid-phase extraction with MCX-Oasis cartridges yielded the highest relative and absolute recoveries of both analytes in bile. Protein precipitation with ACN was used to prepare serum samples, whereas urine samples were used directly after a 20-fold dilution. Alkaline mobile phase (pH 9.3) enhanced both retention time and signal sensitivity, allowing for 1 ng/ml limit of quantification for both analytes. The calibration curves of both analytes were linear over a dynamic range of 1000. This method is useful to study the pharmacokinetics, metabolism,

and disposition of morphine and morphine-3-glucuronide. Our preliminary data shows that M is metabolized to M3G at a lower rate in male than female rats. PB administration induces M metabolism to M3G in male, but not in female WKY rats, and therefore, eliminates the gender difference in M3G levels between male and female rats.

A)



B)

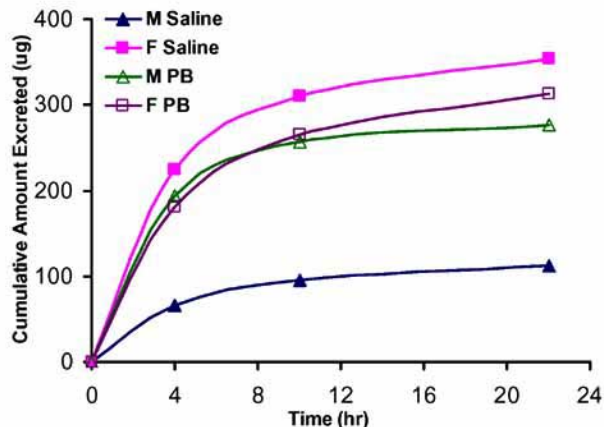


Fig. (9). Cumulative amount excreted in urine vs. time profiles of (A) M and (B) M3G in the 4 treatment groups after subcutaneous administration of M.

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ABBREVIATIONS

LC-MS	=	Liquid chromatography-mass spectrometry
LC-MS/MS	=	Liquid chromatography-tandem mass spectrometry
M	=	Morphine
M3G	=	Morphine-3-glucuronide
IS	=	Internal standard
SPE	=	Solid-phase extraction

ACN	=	Acetonitrile
WKY	=	Wistar-Kyoto
PB	=	Phenobarbital
UGT	=	UDP-glucuronosyltransferase
CAR	=	Constitutive androstane receptor

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