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Simultaneous assay of morphine, morphine-3-glucuronide and morphine-6-glucuronide in human plasma using normal-phase liquid chromatography–tandem mass spectrometry with a silica column and an aqueous organic mobile phase☆

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Abstract

Morphine (MOR) is an opioid analgesic used for the treatment of moderate to severe pain. MOR is extensively metabolized to morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G). A rapid and sensitive method that was able to reliably detect at least 0.5 ng/ml of MOR and 1.0 ng/ml of M6G was required to define their pharmacokinetic profiles. An LC–MS–MS method was developed in our laboratory to quantify all three analytes with the required sensitivity and a rapid turnaround time. A solid-phase extraction (SPE) was used to isolate MOR, M3G, M6G, and their corresponding deuterated internal standards from heparinized plasma. The extract was injected on a LC tandem mass spectrometer with a turbo ion-spray interface. Baseline chromatographic separation among MOR, M3G, and M6G peaks was achieved on a silica column with an aqueous organic mobile phase consisting of formic acid, water, and acetonitrile. The total chromatographic run time was 3 min per injection, with retention times of 1.5, 1.9 and 2.4 min for MOR, M6G, and M3G, respectively. Chromatographic separation of M3G and M6G from MOR was paramount in establishing the LC–MS–MS method selectivity because of fragmentation of M3G and M6G to MOR at the LC–MS interface. The standard curve range in plasma was 0.5–50 ng/ml for MOR, 1.0–100 ng/ml for M6G, and 10–1000 ng/ml for M3G. The inter-day precision and accuracy of the quality control (QC) samples were <7% relative standard deviation (RSD) and <6% relative error (R.E.) for MOR, <9% RSD and <5% R.E. for M6G, and <3% RSD and <6% R.E. for M3G. Analyte stability during sample processing and storage were established. Method ruggedness was demonstrated by the reproducible performance from multiple analysts using several LC–MS–MS systems to analyze over one thousand samples from clinical trials.

Keywords: Morphine; Morphine glucuronides; Opiates

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1. Introduction

Morphine (MOR) is an opiate analgesic frequently used for the treatment of moderate to severe pain. MOR is extensively metabolized via conjugation to morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G) [1]. The analgesic effects of M6G are equal to or more potent than MOR, but with fewer side effects [2]. In contrast, M3G has very little or no analgesic effect by itself [3], but it may be involved in the development of tolerance towards the antinociceptive effects of MOR [4]. The concentration of glucuronide metabolites, particularly M3G, could be much higher than MOR at certain time points [5]. Sensitivity of 0.5 ng/ml for MOR and 1 ng/ml for M6G is required. Simultaneous quantitation of MOR, M3G, and M6G in human plasma is desirable for fast turnaround time.

Opiate analgesics have been successfully analyzed by immunoassays [6–8]. Immunoassays are sensitive, however, these methods may lack the specificity to distinguish the opiates from their corresponding glucuronide metabolites, which may cross-react with the antisera. Antisera produced against an immunogen with conjugation at the *N*-position could provide selectivity against the M3G and M6G. Nevertheless, immunoassays for simultaneous quantitation of all three analytes using multiple antisera would be difficult to develop. Analysis of opiates by GC–MS methods has been reviewed [9,10]. These methods which required derivatization of MOR, had adequate sensitivity for MOR, but GC–MS methods for the analysis of the glucuronide metabolites have not been reported. Capillary zone electrophoresis (CZE) has also been used for the analysis of MOR [11], but sensitivity of the method (1 μg/ml) was inadequate for pharmacokinetic studies. LC methods for the simultaneous analysis of MOR and glucuronide metabolites have been reported, using UV [12], fluorometric [13], electrochemical [14], or mass spectrometric [15–23] detections. UV and fluorometric detections were not sensitive enough for MOR in biological fluids. MOR could be detected by electrochemical detection; however, the chromatographic run time was too long. Most of the reported methods used SPE for sample clean-up. The LC–MS chromatographic run time varied from 5 mins [21] to 25 mins [20]. Only one method used MS–MS detection for all the analytes [22]. None of the published LC–MS methods have achieved both the desired sensitivity (0.5 ng/ml for MOR and 1.0 ng/ml for M6G) and a fast run time (<3 min). We present in this paper the development and validation of an LC–MS–MS method to achieve these goals.

2. Experimental

2.1. Chemicals and reagents

MOR sulfate·$5H_2O$, hydromorphone hydrochloride (HYD·HCl), buprenorphine hydrochloride, naloxone, and hydrocodone bitartrate were from United States Pharmacopeia (Rockville, MD, USA); M3G (purity >99%) was from Sigma (St. Louis, MO, USA); and M6G·$3H_2O$ (purity 99.9%) was from RBI (Natick, MA, USA). Internal standards (I.S.) MOR-d_3 and M6G-d_3 were from High Standard Products Corporation (Inglewood, CA, USA) and M3G-d_3 was from Isotec (Miamisburg, OH, USA). Acetonitrile, methanol and water were of HPLC grade and were from Fisher (St. Louis, MO, USA). Trifluoroacetic acid (TFA) was from Sigma and formic acid was from Mallinckrodt (Irvine, CA, USA). Control human heparinized plasma was from Nashville Biologicals, (Cincinnati, OH, USA) or from our in-house collection. Bond Elut C_{18} SPE cartridges (3cc, 200 mg) were from Varian (Harbor City, CA, USA).

2.2. LC–MS–MS

The LC–MS–MS system consisted of a Jasco pump (Tokyo, Japan), a Waters 717 plus autosampler (Milford, MA, USA), and a PE Sciex 365 MS–MS (Torondo, Canada) with Turbo IonSpray® connected to an Inertsil silica column of 5 μm, 50×3 mm I.D. from Keystone (Bellefonte, PA, USA). The injection volume was 5 μl; run time was 3 min; flow-rate was 1.0 ml/min. Analytical columns from five different batches have been used and consistent, similar chromatographic performance was observed. The column was maintained at ambient temperature. After 5–10 min of equilibration with a mobile phase consisting of formic acid–water–acetonitrile (1:10:90, v/v/v), the new silica columns showed

consistent retention times of the analytes. Without any treatment, one column could be used for at least five hundreds injections of the extracted samples.

Sensitivities of multiple reaction mode (MRM) were optimized by testing with an infusion of 1 μg/ml MOR or M6G in a mixture of methanol and water (50:50, v/v). The drying gas (nitrogen) flow-rate of the Turbo IonSpray® was 8 l/min. The electrospray source was operated with a capillary voltage of 4.5 kV, an orifice voltage of 35 V, and a source temperature of 350°C. The ring voltage was 240 V, and Q1 energy was −0.6 eV. The preselected protonated precusor ion masses, which passed the first MS, went into the collision cell. The fragmentation occurred at collision energies of −48 eV and −38 eV for MOR and M3G/M6G, respectively. Nitrogen was used as collision gas. The product ions with the preselected masses produced from the fragmentation in the collision cell passed the second MS and were detected. The Q3 energy was −3 eV. The dwell time was 500 ms for the analytes and 200 ms for I.S. The following ions (precursor to product) were monitored: MOR, $m/z=286\rightarrow165$; M3G, $m/z=462\rightarrow286$; M6G, $m/z=462\rightarrow286$; MOR-d_3, $m/z=289\rightarrow165$; M3G-d_3, $m/z=465\rightarrow289$; M6G-d_3, $m/z=465\rightarrow289$. For MOR and MOR-d_3, several product ions were observed but the most abundant one was chosen. For the metabolites, only one product ion was observed.

Chromatograms were integrated using Apple PowerMac® 8600/200 and the data transferred to the VAX®/VMS for regression. A weighted $1/x^2$ linear regression was used to determine slopes, intercepts and correlation coefficients (r^2), where x is the concentration of the analyte. The resulting parameters (y-intercept and slope) were used to calculate concentrations from the equation: Concentration=[ratio−(y-intercept)]/slope, where 'ratio' is the ratio of the compound peak area to the I.S. peak area.

2.3. Calibration standards and quality control samples

Two sets of primary stock methanolic solutions of MOR, water solutions of M3G and M6G, were prepared from separate weighings for standard and quality control samples (QCs). Working standards were prepared fresh daily by spiking 100 μl of ten-fold concentrated aqueous solutions, containing all three analytes, into1.0 ml of blank control heparinized plasma. The final concentrations in plasma standards were 0.50/1.00/10.0, 1.00/2.00/20.0, 2.00/5.00/50.0, 5.00/10.0/100, 10.0/20.0/200, 20.0/40.0/400, 40.0/80.0/800, and 50.0/100/1000 ng/ml for MOR/M6G/M3G. Three levels of QCs in human plasma, 1.50/3.00/30.0, 15.0/15.0/150, and 35.0/75.0/750 ng/ml for MOR/M6G/M3G were prepared, aliquoted and stored frozen at −20°C.

2.4. Sample preparation

To 1.0 ml of plasma sample in a silanized glass tube, 100 μl of an aqueous I.S. solution (250/500/1000 ng/ml for deuterated MOR/M6G/M3G) and 1.0 ml of 0.1% TFA in water were added. After mixing, samples were applied to the C_{18} SPE cartridges which had been conditioned with 3 ml of methanol, 3 ml of water, and 3 ml of 0.1% TFA in water. The cartridges were connected to a Baker SPE-10 vacuum manifold (Phillipsburg, NJ). Samples were allowed to pass through cartridges by gravity. The cartridges were then washed with 4 ml of 0.1% TFA in water. The analytes were eluted with 2 ml of methanol–water (50:50, v/v) into silanized glass tubes. The eluent was evaporated to dryness at 55°C in a Turbo Vap® LV Evaporator from Zymark (Hopkinton, MA) under a stream of nitrogen. The residue was reconstituted in 150 μl of acetonitrile containing 0.1% (v/v) of formic acid by vortex-mixing at high speed for 2 min and transferred to autosampler vials.

2.5. Validation of the LC–MS–MS method

The method was validated by five analytical curves on five separate days. Each calibration curve contained a single set of calibration standards and six replicates of QCs at each concentration level. Each curve also contained other test samples such as stability samples of processing and storage. QCs were randomized through the curve among the samples. One curve contained over 100 samples to simulate the length of a clinical sample analysis run.

Analyte stability was tested by subjecting QCs through multiple freeze–thaw cycles (F/T cycles),

on the bench at room temperature (bench-top), or at –20°C in the freezer for a long period (storage). Post-extraction analyte stability (refrigeration and reinjection) was determined by comparing the results to those of freshly extracted samples.

Recoveries of the analytes were determined by comparing the peak area of the analytes extracted from plasma with those in unextracted solution which was prepared by spiking analytes into plasma extracts (post-extraction). The recovery testing was performed at three levels (the highest standard, a middle standard and the lowest standard).

The method selectivity was evaluated by screening eight lots of blank control human heparinized plasma, including two lots of lipemic plasma. These lots were spiked with known concentrations of MOR, M3G and M6G, extracted, and injected onto LC–MS–MS to determine the consistency of between-lot recoveries. Control plasma samples were also spiked separately with either MOR, M3G or M6G and extracted to determine any potential conversion of M3G and M6G to MOR during the sample extraction process.

The method ruggedness was evaluated from calibration curves performed by multiple analysts on multiple LC–MS–MS instruments with different lots of the analytical columns.

3. Results and discussion

3.1. ESI mass spectra

Figs. 1 and 2 show the full scan mass spectra of product ions for MOR and M6G, respectively. The full scan mass spectrum of product ion for M3G was identical to that for M6G. M6G and M3G had the same precusor ion at m/z 462 and deconjugation product ions at m/z 286. MOR fragmented to multiple product ions. We chose the product ion (m/z 165) that was of the highest intensity. This product ion was different from the one (m/z 152) chosen by Zheng et al. [22]. It should be mentioned that the method specificity could be further confirmed by monitoring multiple product ions or ion ratios, which is frequently used in forensic drug testing. However,

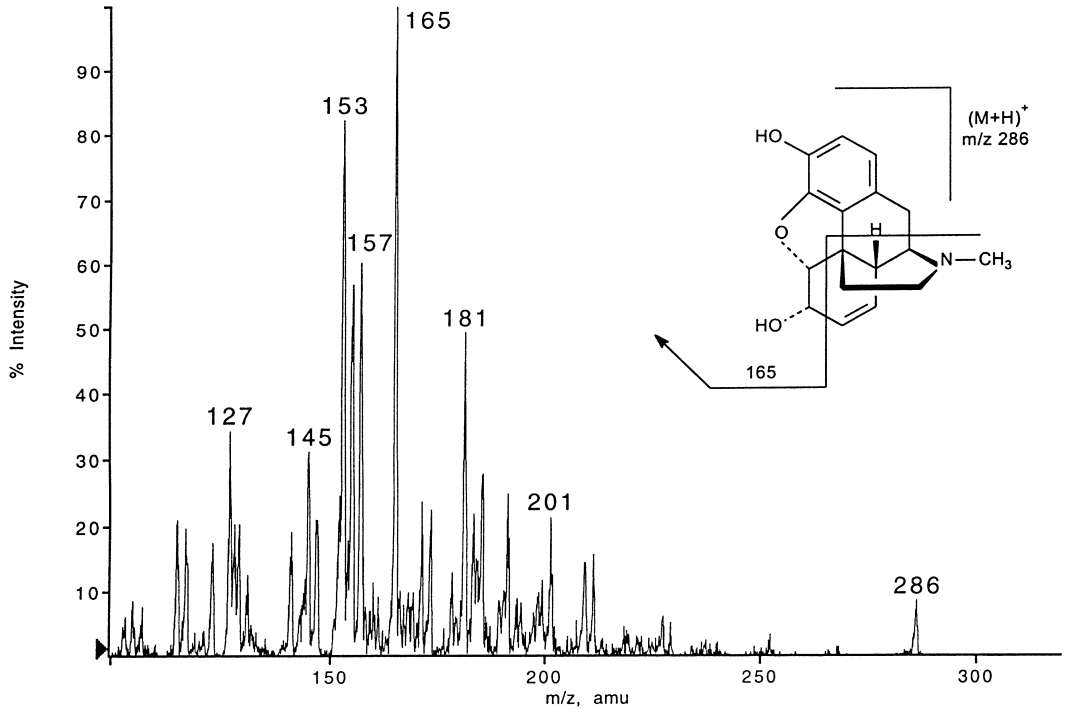

Fig. 1. ESI–MS–MS spectrum of MOR.

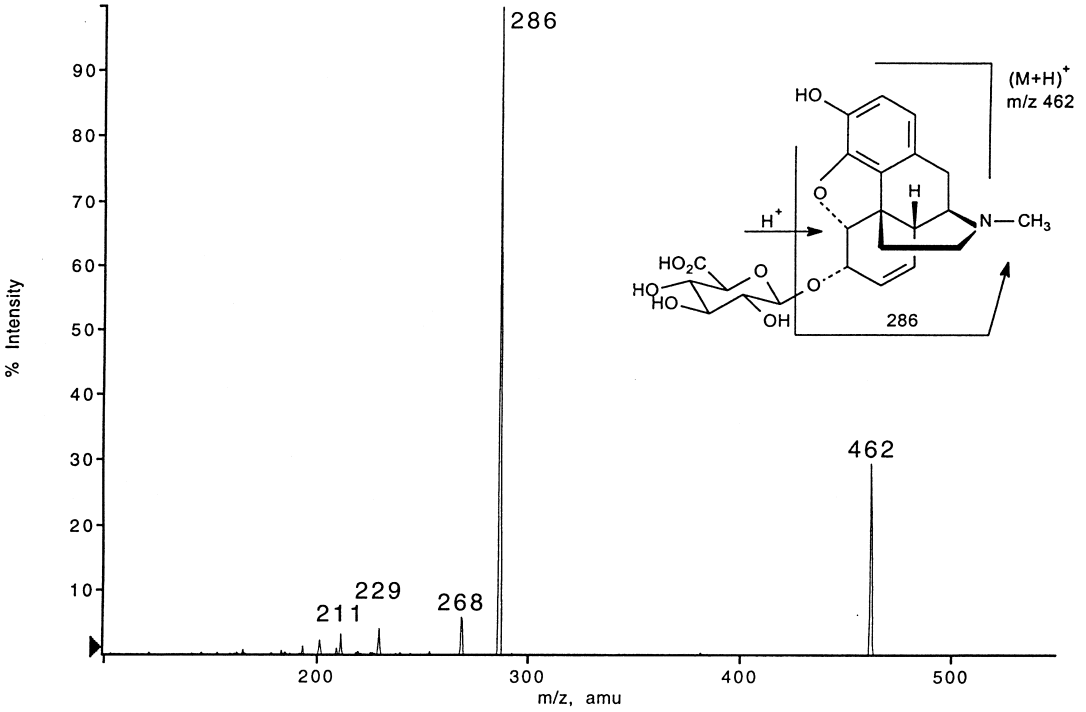

Fig. 2. ESI–MS–MS spectrum of M6G.

the dwell time for each product ion would have to be decreased and this could lead to lower sensitivity in comparison with single product-ion monitoring.

3.2. Chromatography

One of the primary goals set at the beginning of our study was to separate M3G from M6G because they have identical m/z and product ions, and equally important, to separate MOR from its glucuronide metabolites. The conjugated metabolites could fragment to MOR in the LC–MS interface and be falsely detected as MOR if the separation was not achieved [24]. With the reversed-phase HPLC separation, a mobile phase of low organic content is required to retain the analytes on the column. Chromatographic separation of the analytes from the major matrix materials as indicated by the retention on the column ($k'>1$), is also important to alleviate severe matrix suppression [25–29]. A mobile phase of high aqueous/low organic components should therefore be used for reversed-phase LC–MS–MS in order to retain these analytes on the column. However, mobile phases with little organic modifier adversely affect sensitivity, due to the poor spray condition that results from high surface tension of the aqueous droplets. This is especially true when a conventional flow-rate (in 0.1 ml/min to 1 ml/min range) and a turbo ESI interface is used [30]. The temperature of the drying gas has to be increased to obtain a good spray to improve sensitivity. However, an elevated temperature would cause more undesirable in-source fragmentation of M3G and M6G. Furthermore, protonated ions of MOR, M3G and M6G in an acidic mobile phase were recommended for the good sensitivity [31]. However, the protonated MOR, M3G and M6G are even more polar than their corresponding unprotonated species, and an even lower organic concentration in the mobile phase would be required to retain them on the column. Therefore, the mobile phase of high organic content and acidic pH was required to produce good spraying condition and sensitivity for MS. However, this kind of mobile phase in reversed-phase chromatography would not provide the column retention and resolution, which are needed to avoid matrix suppres-

sion and glucuronide fragmentation artifacts. Instead of reversed-phase chromatography, we used normal-phase LC–MS–MS for the analysis of MOR and its glucuronide metabolites.

Normal-phase LC (NPLC) uses a stationary phase that is relatively more polar than the mobile phase. Mobile phases used in traditional normal-phase consist of a very non-polar solvent such as hexane and small amounts of polar organic solvents such as ethanol. They are used to separate very non-polar compounds such as steriods. The level of trace amount (in the ppm range) of water in the mobile phase has to be stringently controlled [32]. Slight changes in the water content cause changes in peak shape and retention. We investigated the feasibility of separating MOR, M3G and M6G in human plasma by NPLC–MS–MS using an aqueous organic mobile phase on a silica column instead of the traditional solvents of NPLC. The mobile phase consisted of mainly acetonitrile, a substantial amount of water, and formic acid. Formic acid was used to improve the analyte ionization and peak shape. Instead of long equilibration time, an extremely fast equilibration time of 5–10 min can be achieved for a new column. Previous NPLC using methanol as the mobile phase major component requires a long equilibration time; M3G and M6G were eluted in front of MOR [20]. The elution order indicated that reversed-phase retention mechanism might play a role here because the more polar M3G and M6G should be eluted in front of MOR under true normal-phase conditions. This reversed-phase separation mechanism on silica column probably could be attributed to the hydrophobic siloxane groups on the silica surface [33]. Chromatographic methods on the silica column using a mobile phase of methanol and an aqueous buffer at neutral to alkaline pH involved a complicated separation mechanism of ion-exchange, ion-pair, salting-out, and reversed-phase retention [34–37]. A strong interaction between methanol rather than acetonitrile and silanol groups of the silica support has been reported [38]. Methanol is a much stronger elution solvent than acetonitrile on silica column, because of its stronger interaction with the silanol groups. Therefore, with a mobile phase consisting of methanol and aqueous solution, both being very strong eluting solvents, a reversed-phase rather than normal-phase mechanism could predominate. In other words, the mobile phase is probably more polar than the silica stationary phase. Under this condition, the reversed-phase mechanism due to the hydrophobic silaxone groups governs the column retention of the analytes. Methanol-modified silica surfaces were reported to be less homogeneous than water-modified silica [39]. Methanol and water might compete for the interaction with silanol groups, resulting in a long equilibration time. On the other hand, a mobile phase consisting only of acetonitrile and water contains one polar component and column equilibration can be very fast. With acetonitrile in the mobile phase, the retention time was very reproducible and the column did not need to be conditioned for a long time. No additional conditioning of the column was required between the runs. The influence of mobile phase composition and column temperature on the separation was investigated. Increasing the water content shortened the retention time as predicted by a normal-phase mechanism. Altering the column temperature had almost no effect on the analytes' retention times. Doubling the formic acid concentration slightly decreased the analytes' retention times. The adsorption activities of the silanol groups on the silica surface could be decreased by increasing the acidity of the mobile phase. For the same reason, when formic acid was replaced by acetic acid, the retention time increased. The silica column demonstrated excellent column stability. At least five hundred extracted samples could be injected onto the same column without loss of performance. Fig. 3 shows the chromatograms of initial injection and injection after 300 sample analyses. No column performance deterioration was noted. The resolution of MOR/M6G and M6G/M3G remained at 2.5 and 2.4, respectively. The NPLC–MS–MS using silica column with aqueous organic mobile phase had been used in our laboratory for the bioanalysis of over twenty compounds including nucleosides, drugs with multiple polar functional groups, acidic polar compounds, basic compounds, and conjugated metabolites [40]. This NPLC–MS–MS system was compatible with the various extraction techniques including protein precipitation, dilute and shoot, liquid–liquid extraction, and SPE. Column stability was excellent

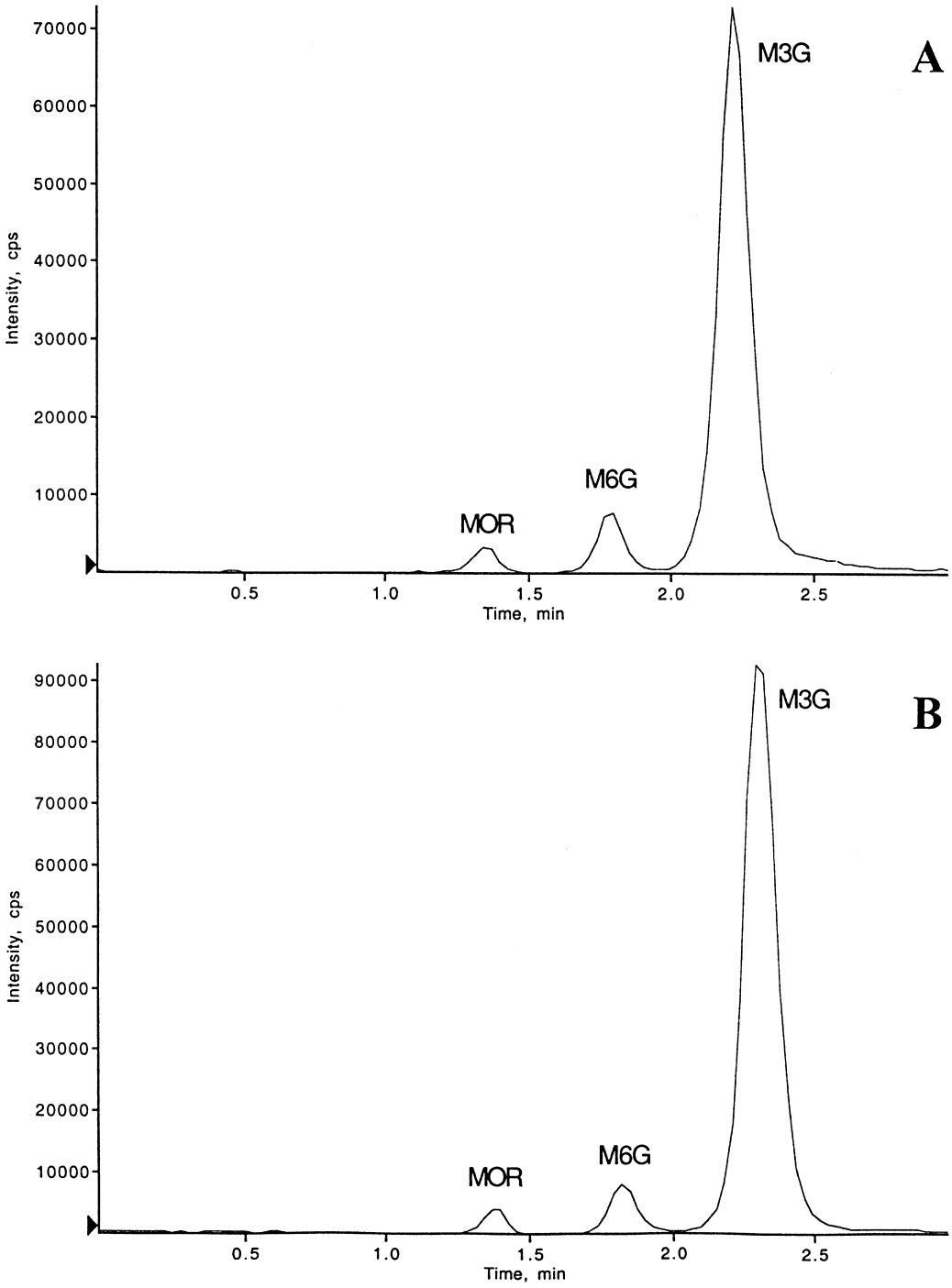

Fig. 3. Silica column stability: Total ion current traces of MOR, M3G and M6G of the human plasma spiked with 50 ng/ml MOR, 100 ng/ml M6G, and 1000 ng/ml M3G.. (A) Injection #12; (B) injection #303.

even with injection of samples from protein precipitation.

3.3. Post-column conversion of M3G and M6G to MOR

Our group and others have observed post-column deconjugation by fragmentation of hydromorphone-3-glucuronide to hydromorphone [24] as well as M3G and M6G to MOR [21,24]. This deconjugation in the LC–MS interface can lead to false over-estimation of the parent compound. Fig. 4 shows the chromatogram of post-column deconjugation of M3G and M6G to MOR. Three peaks were observed at the MOR m/z channel with their retention times corresponding to MOR, M6G and M3G, respectively. The deconjugated product of M3G and M6G passed the MS–MS detectors in the same manner as MOR and appeared at the MOR m/z channel. It was estimated about 2% M3G and M6G were deconjugated to the product of the same MOR m/z in the interface. This minor break down of M3G and M6G will not affect the accurate quantitation of these two metabolites. However, if not separated from MOR, 2% deconjugation of M3G was large enough to cause about 50% over-estimation of MOR because M3G concentration in plasma can be 25 times higher than MOR. Attempts to completely eliminate the deconjugation by changing interface temperature and voltage were unsuccessful. Poorer sensitivity was observed at a lower interface temperature and voltage. Therefore, chromatographic separation of the glucuronide-conjugated metabolites from parent compounds is important to establish the selectivity of the LC–MS–MS method.

3.4. Extraction and recovery

Solid-phase extraction was reported in literature methods to extract MOR, M3G and M6G simultaneously. Prior to being loaded to the reversed-phase SPE cartridges, the samples were buffered to pH 9.3 with ammonium acetate [15], potassium carbonate [18], or ammonium carbonate [20–22]. Both reversed-phase retention and secondary interactions may play roles in the retention mechanism of the analytes [18]. A tight control of the pH of the buffer was needed for these methods. Here we developed a simple ion-pairing SPE. M3G and M6G are polar compounds, which may not be well retained on C_{18} cartridge. In order to decrease the polarity, TFA was added to form an ion-pair with the analytes. The recovery was 88% for MOR, 70% for M3G, and 93% for M6G. These results were comparable to the published data using an alkaline buffer. Though suppression of the LC–MS–MS signal due to ion pairing with TFA has been reported and post-column addition of a weaker acid such as propionic acid was used to improve sensitivity [41], signal suppression was not observed in our method using TFA in SPE. This is probably due to the fact that TFA was not used in the mobile phase. The minute amount of TFA left in the sample extract, if there is any, would be eluted at the solvent front and would not form an ion-pair with the anaytes. The experiment result showed that the addition of 0.01% TFA in the sample extract did not cause any signal suppression. A recent publication used solid-phase immuno-extraction to isolate MOR from urine samples [42]. Immunoaffinity extraction in conjunction with LC–MS–MS certainly is a research area that warrants more attention [43]. However, antisera from multiply immunogens or broad-spectrum antisera will be required to bind MOR, M3G and M6G for the simultaneous extraction of all three analytes, which may require more research and development time than our method.

3.5. Selectivity and sensitivity

Eight lots of blank control plasma, including two lipemic lots, were tested for matrix interference. They did not show interfering peaks at the retention time of the compounds of interest. Method selectivity was further confirmed by the lack of interference peaks from over 100 pre-dose clinical samples. Method selectivity was also tested against other structurally similar compounds. Oxymorphone, naloxone, buprenorphine and hydrocodone did not interfere with the MOR peak since they have m/z different from MOR. Hydromorphone (HYD), which has the same m/z as MOR and could be observed at the MOR channel, was partially separated from

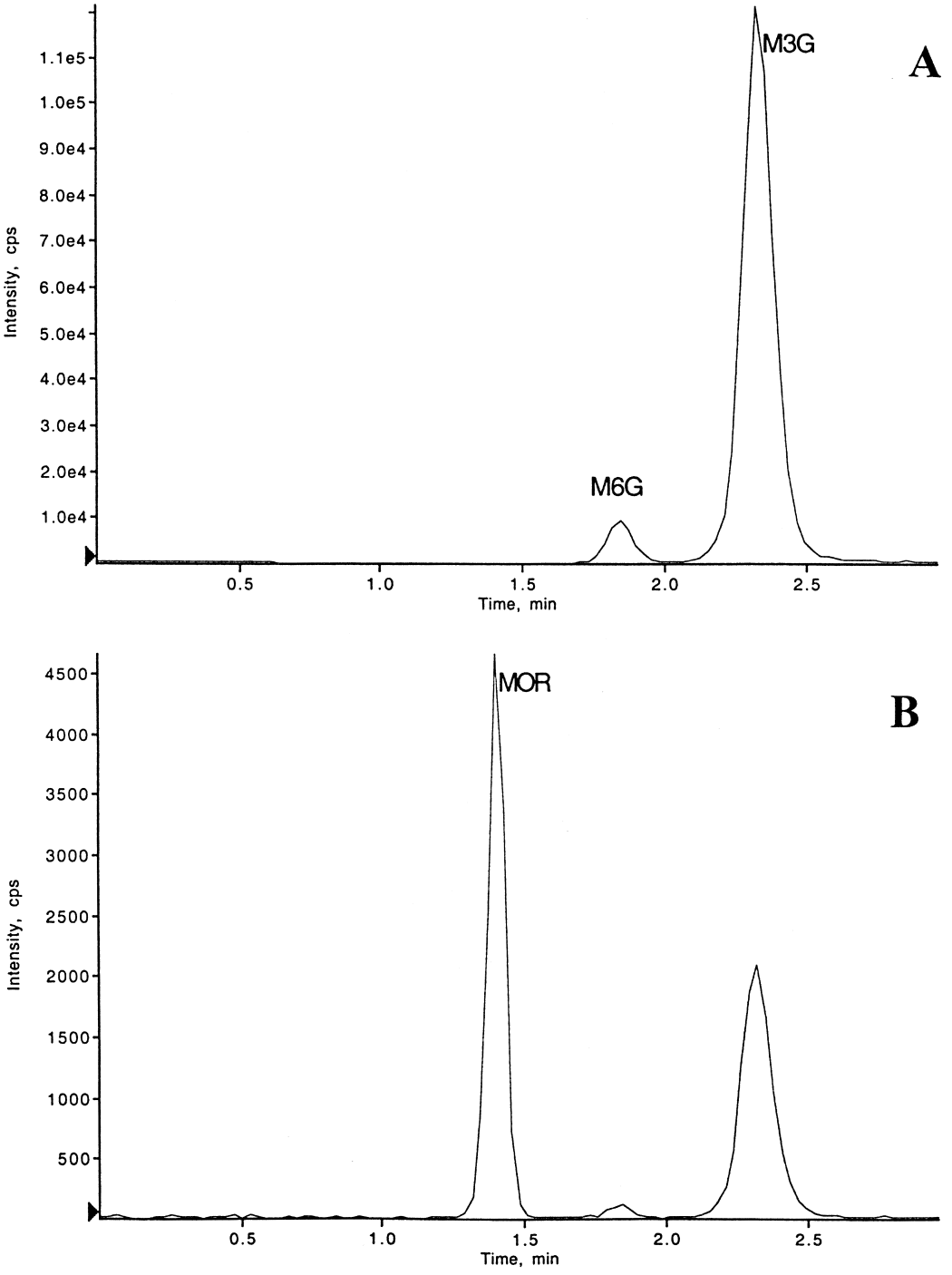

Fig. 4. Fragmentation of M3G and M6G to MOR in LC–MS interface. Human plasma was spiked with 50 ng/ml MOR, 100 ng/ml M6G, and 1000 ng/ml M3G. (A) M3G and M6G channel (462→286); (B) MOR channel (*m*/*z* 286→165). The two extra peaks observed in the MOR channel have a retention time corresponding to M3G and M6G.

MOR peak. The retention time for HYD and MOR is 1.2 and 1.3 min, respectively. Resolution of HYD and MOR was improved by using a mobile phase of formic acid–water–acetonitrile (1:5:95, v/v/v). HYD, MOR, M6G and M3G elute at 2.0, 2.3, 3.1 and 4.3 min, respectively. Complete resolution of HYD and MOR would be desirable in forensic studies. However, it is not needed for the intended pharmacokinetic study. When the samples were spiked with the analytes at their limit of quantitaion (LOQ), which was 0.5/1/10 for MOR/M6G/M3G, the observed concentrations were within 20% of the theoretical values. The relative standard deviations (RSDs) were 10.6/2.6/14.1% for MOR/M3G/M6G of the spiked LOQ samples. No conversion from M3G or M6G to MOR was observed during the extraction. The individually extracted M3G (1000 ng/ml) and M6G (100 ng/ml) plasma samples did not show an increased MOR concentration, compared with unextracted M3G and M6G solutions which was prepared by diluting M3G and M6G stock solutions with acetonitrile. No response was observed at the M6G and I.S. channels for the extracted M3G sample and no response was observed at the M3G and I.S. channels for the extracted M6G sample. The results indicated that M3G or M6G was not degraded during the sample extraction and chromatography processes. The minute amount of MOR ($<0.1\%$) in M3G and M6G did not cause bias in MOR determination.

Fig. 5 shows the chromatograms of human plasma spiked with 0.5 ng/ml MOR, 1 ng/ml M6G, and 10 ng/ml M3G and blank plasma. Fig. 6 shows the chromatogram of human plasma spiked with the I.S. only. Lack of any response from the I.S. into the MOR/M3G/M6G channels was demonstrated. The signal-to-noise ratio for MOR is about 7 at 0.5 ng/ml. No interferences were observed in the blank plasma. The lowest concentration in the standard range was defined as the limit of the quantitation (LOQ) because these concentrations were adequate to define the elimination profile of the analytes for the intended pharmacokinetic studies. They may not be the lowest concentrations that can be quantified reliably. The estimated limit of detection (LOD) at a signal-to-noise ratio of 3 was about 0.25 ng/ml for MOR and M3G, and 0.5 ng/ml for M6G.

3.6. Precision, accuracy and linearity

Table 1 shows the validation data on accuracy and precision of each standard concentration. The correlation coefficients of the five validation curves were all >0.99. The low RSD values for the slope of each analyte indicated reproducible LC–MS–MS instrument conditions. The standards show a linear range of 0.5–50 ng/ml for MOR, 10–1000 ng/ml for M3G, and 1–100 ng/ml for M6G. The choice of the regression methods was evaluated. Both MOR and M6G had a better fit using $1/x^2$ regression mode. Lower RSDs were observed with $1/x^2$ regression than those by $1/x$ regression mode. For MOR, the relative response factors (ratio of analyte area vs. I.S. area divided by analyte concentration) decreased slightly towards the LOQ, while that increased. A $1/x^2$ regression mode would fit better than the $1/x$ mode. There was little difference between $1/x$ and $1/x^2$ for M3G. For consistency sake, all three analytes were regressed using $1/x^2$.

Table 2 presents the inter-day and intra-day accuracy and precision of QCs. The data show that this method is consistent and reliable with low values of RSDs and R.E.s.

3.7. Stability of the analytes

The protocol of the stability tests was designed to cover the anticipated conditions that the clinical samples may experience. Stabilities of sample storage (long-term storage), processing (freeze–thaw, bench-top, and refrigeration) and chromatography (re-injection) were tested and established. The results are summarized in Table 3.

4. Application

The normal-phase LC–MS–MS method developed here was used to study the pharmacokinetic profiles of MOR and its metabolites. The drug concentration versus time profiles in plasma from a volunteer dosed orally with a 30-mg tablet of MOR sulfate is shown in Fig. 7. Peak plasma concentrations were 10, 50, and 250 ng/ml for MOR, M6G, and M3G, respectively. Over 1000 samples were analyzed.

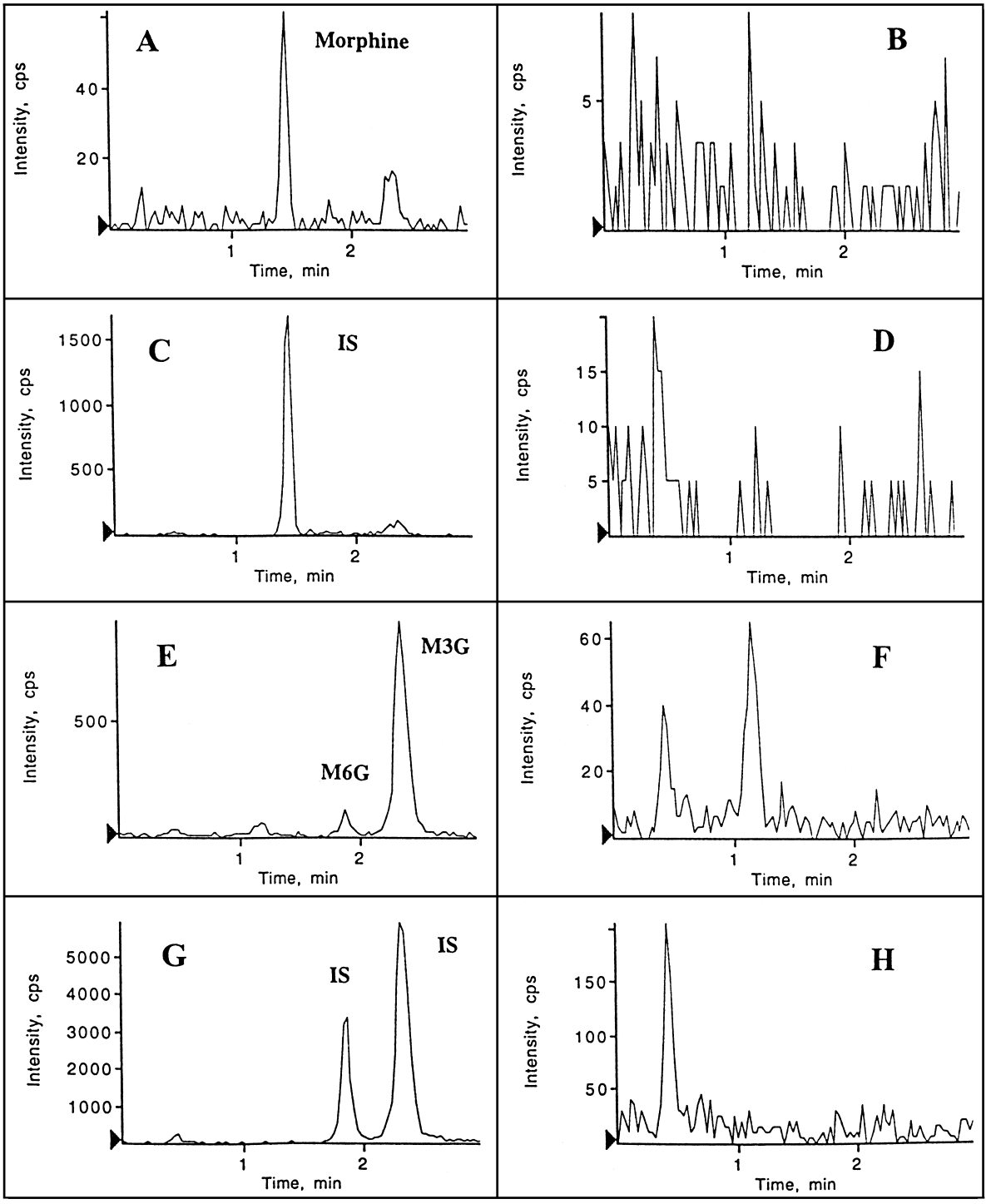

Fig. 5. NPLC–MS–MS chromatograms of human plasma spiked with MOR, M6G, and M3G and blank plasma. (A) MOR at 0.5 ng/ml; (B) MOR in blank plasma; (C) MOR-d_3 (I.S. for MOR); (D) MOR-d_3 in blank plasma; (E) M6G at 1.0 ng/ml and M3G at 10 ng/ml; (F) M6G and M3G in blank plasma; (G) M6G-d_3 and M3G-d_3 (I.S. for M6G and M3G); (H) M6G-d_3 and M3G-d_3 in blank plasma.

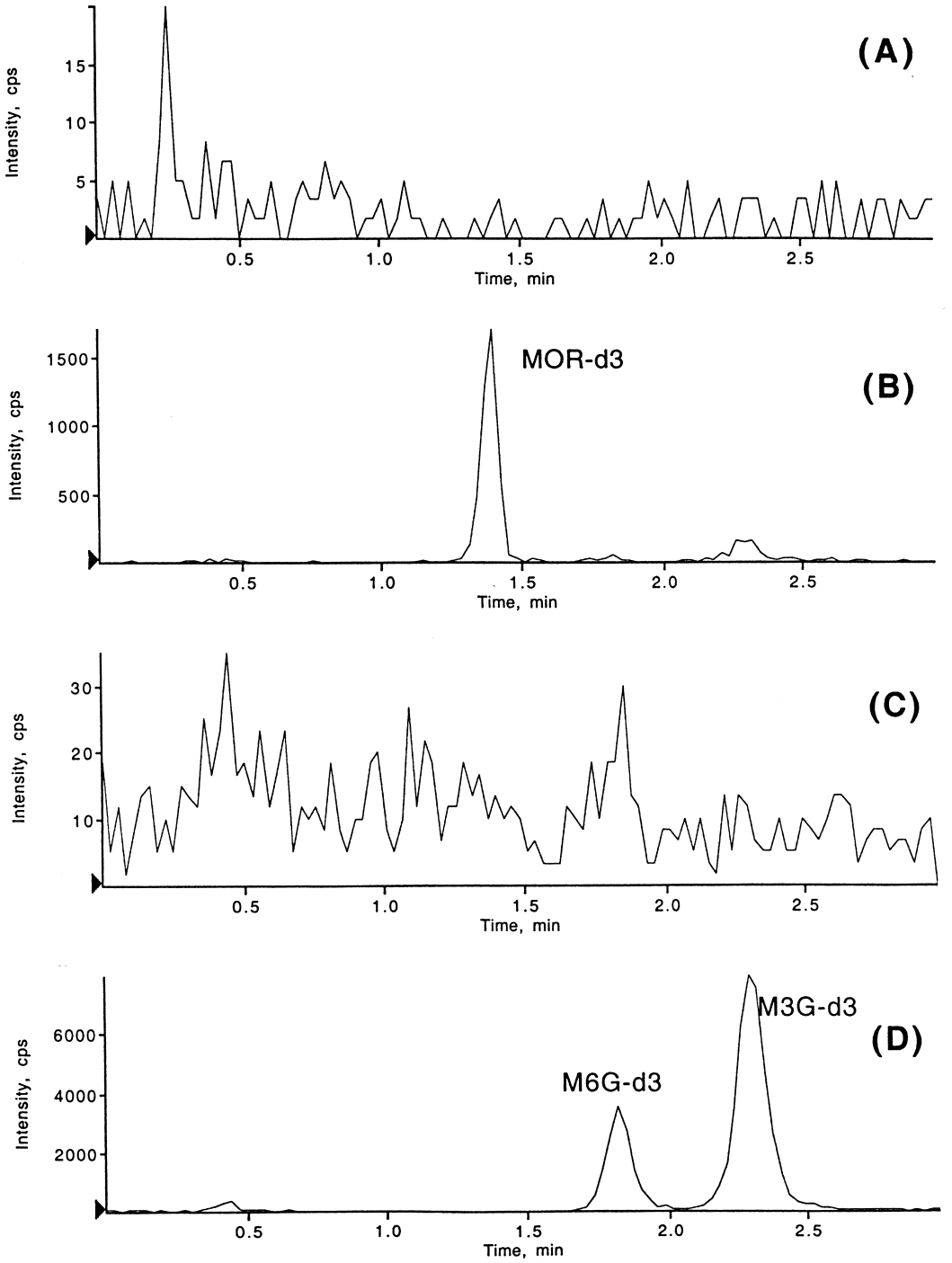

Fig. 6. NPLC–MS–MS chromatograms of human plasma spiked with I.S. only. (A) MOR; (B) MOR-d_3; (C) M6G and M3G; (D) M6G-d_3 and M3G-d_3.

Table 1
Linearity of calibration standards from five validation curves

	Standard concentration (ng/ml)									
MOR	0.50	1.00	2.00	5.00	10.0	20.0	40.0	50.0	Slope	r^2
Mean	0.48	1.08	1.96	4.87	9.95	20.4	39.5	49.6	0.0653	0.9976
%RSD	2.08	4.63	7.14	5.54	2.71	3.18	6.23	5.37	1.99	0.13
%R.E.	−4.00	+8.00	−2.00	−2.60	−0.50	+2.20	−1.25	−0.90		
M3G	10.0	20.0	50.0	100	200	400	800	1000		
Mean	10.0	20.0	49.7	98.7	210	404	793	970	0.0138	0.9995
%RSD	1.20	2.05	1.91	2.05	1.45	1.56	1.43	1.14	2.17	0.03
%R.E.	+0.10	−0.15	−0.64	−1.33	+4.88	+1.04	−0.86	−2.97		
M6G	1.00	2.00	5.00	10.0	20.0	40.0	80.0	100		
Mean	1.01	1.90	5.33	9.98	20.6	39.2	78.6	98.2	0.0243	0.9981
%RSD	2.97	6.84	4.88	4.81	2.53	2.86	2.10	3.75	5.76	0.13
%R.E.	+1.00	−5.00	+6.60	−0.20	+2.85	−2.12	−1.74	−1.79		

Concentration of the analytes in all of the 50 pre-dose samples was well below the LOQ.

5. Conclusion

A non-traditional normal-phase LC–MS–MS method was developed for the simultaneous analysis of morphine and its two glucuronide-metabolites with a 3-min run time. With a silica stationary phase and acetonitrile–water–formic acid as mobile phase, all three analytes were baseline-resolved. Such baseline resolution was important to avoid artificial overestimation of morphine from deconjugation of M3G in the LC–MS interface. The LOQ was 0.5 ng/ml for MOR and 1.0 ng/ml for M6G in plasma. One analyst could process approximately 200 samples per day and over 400 samples per day can be analyzed on one instrument. Method ruggedness was

Table 2
Precision and accuracy of quality control samples

	Quality control sample concentration (ng/ml)					
	Intra-day (n=6)			Inter-day (n=30)		
MOR	1.50	15.0	35.0	1.50	15.0	35.0
Mean	1.55	14.7	35.1	1.58	14.7	34.8
%RSD	6.45	6.72	2.79	6.33	5.93	4.85
%R.E.	+3.33	−1.73	+0.23	+5.33	−2.27	−0.54
M3G	30.0	150	750	30.0	15.0	35.0
Mean	32.3	155	759	31.5	155	757
%RSD	2.51	2.00	2.78	2.98	2.76	2.72
%R.E.	+7.63	+3.32	+1.15	+5.13	+3.35	+0.91
M6G	3.0	15.0	75.0	3.00	15.0	75.0
Mean	3.27	16.4	76.4	3.12	15.5	75.4
%RSD	7.95	2.43	3.47	8.97	5.03	4.02
%R.E.	+9.00	+9.53	+1.91	+4.00	+3.33	+0.51

Table 3
Stability of quality control samples during storage, sample processing and chromatography[a]

	Period			Percentage of control		
	MOR	M3G	M6G	MOR	M3G	M6G
Plasma sample						
Bench top	52 h	25.5 h	25.5 h	98–102	100–101	98–103
F/T cycles	6	4	4	97–101	99–101	99–101
Storage	19 m	15 m	15 m	103–104	98–101	94–112
Sample extract						
Re-injection (2–8°C)	133 h	133 h	133 h	97–105	98–101	92–98
Refrigeration (2–8°C)	93 h	93 h	93 h	95–100	101–104	98–106

[a] h: hour; m: month.

demonstrated by multiple analysts and on multiple LC–MS–MS systems. This method was validated to meet the pharmaceutical industry guideline [44]. Linearity, precision and accuracy were demonstrated. Recoveries, stability of storage, freeze–thaw cycles, bench-top, and re-injection were established. The

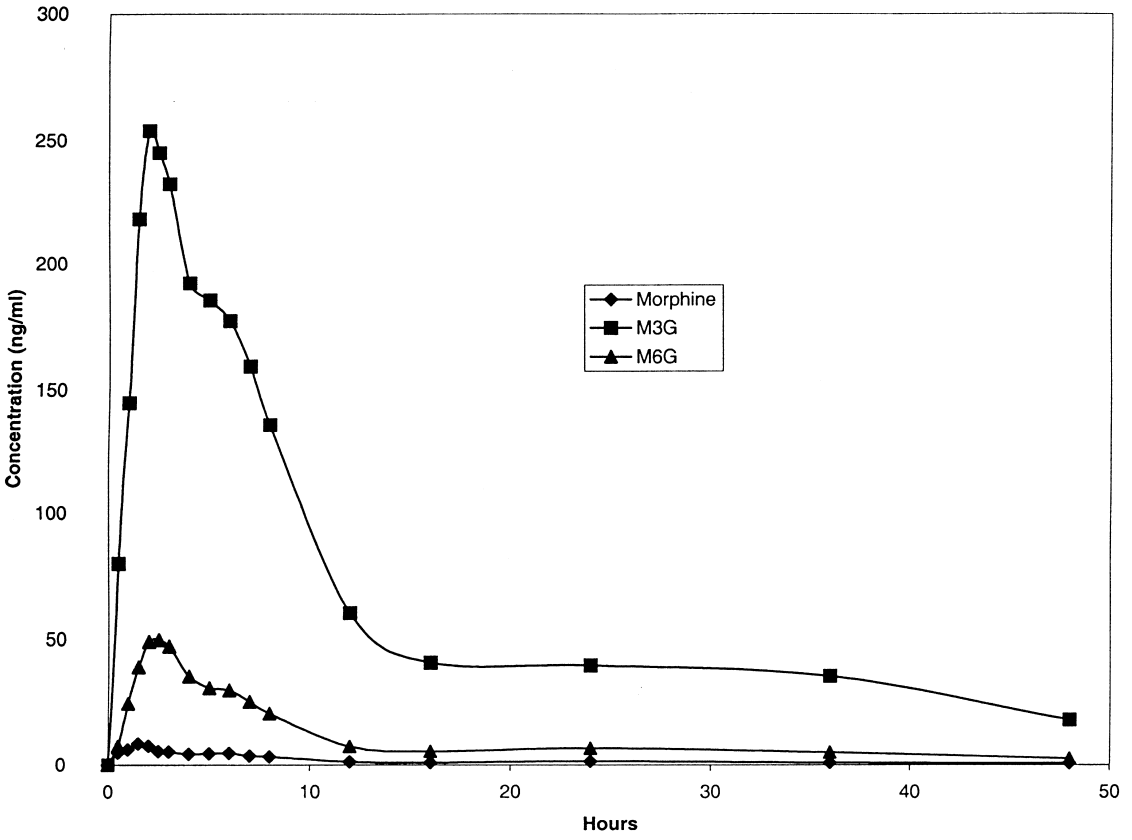

Fig. 7. Concentration–time profile from a healthy volunteer dosed orally with a 30-mg morphine sulfate tablet.

method has been successfully applied to analysis of over 2000 samples from clinical trials.

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