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An automatic 96-well solid phase extraction and liquid chromatography–tandem mass spectrometry method for the analysis of morphine, morphine-3-glucuronide and morphine-6-glucuronide in human plasma

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Abstract

A bioanalytical method using automated sample transferring, automated solid phase extraction (SPE) and liquid chromatography–tandem mass spectrometry (LC-MS-MS) was developed for morphine (MOR), and its metabolites morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G) in human plasma. Samples of 0.25 ml were transferred into 96-well plate using automatic liquid handler (Multiprobe™ II). Automated SPE was carried out on a 96-channel programmable liquid handling workstation (Quadra™ 96) using a C_{18} sorbent. The extract was injected onto a silica column using an aqueous-organic mobile phase. The chromatographic run time was 3.5 min per injection, with retention times of 1.5, 2.0 and 2.6 min for MOR, M6G, and M3G, respectively. The detection was by monitoring MOR at m/z 286→152, M6G and M3G at m/z 462→286. The deuterated internal standards were monitored at m/z 289→152 for MOR-d_3, and m/z 465→289 for M6G-d_3 and M3G-d_3. The standard curve range was 0.5–50 ng ml^{-1} for MOR, 1.0–100 ng ml^{-1} for M6G, and 10–1000 ng ml^{-1} for M3G. The inter-day precision and accuracy of the quality control samples were $<8\%$ relative standard deviation (RSD) and $<7\%$ relative error (RE) for MOR, $<5\%$ RSD and $<2\%$ RE for M6G, and $<2\%$ RSD and $<4\%$ RE for M3G.

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

1. Introduction

Morphine (MOR), an opiate analgesic, is extensively metabolized to form phase II metabolites morphine-6-glucuronide (M6G) and morphine-3-glucuronide (M3G) [1]. The analgesic effects of M6G are equal to or more potent than MOR, but with fewer side effects [2,3]. M3G has little analgesic effect but it may play a role in the development of tolerance towards the antinociceptive effects of MOR [4,5]. The plasma concentration of M3G could be much higher than MOR [6,7].

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For fast turnaround time, simultaneous quantitation of MOR, M6G and M3G at low ng ml^{-1} level (0.5 ng ml^{-1} for MOR, 1 ng ml^{-1} for M6G, and 10 ng ml^{-1} for M3G) in human plasma is needed.

Earlier analytical methods for the quantitation of MOR and metabolites have been reviewed in a previous publication [8]. Only two LC-MS-MS methods described simultaneous analysis of MOR and metabolites [8,9]. While the method reported by Zheng et al. [9] lacked the required sensitivity, the method by Naidong et al. [8] was time-consuming due to manual solid phase extraction (SPE) extraction using gravity elution.

In this paper, we present a highly automated LC-MS-MS method for the simultaneous assay of MOR, M6G and M3G in human plasma. This method was developed based on a previously published method [8] but particular effort was made to automate the sample preparation step. As a direct result of the short analysis times offered by LC-MS-MS, sample preparation has become the rate-limiting step [10]. Much effort has been devoted to automate the sample preparation step by using 96-well plate format [11–14]. Therefore, the method described here also utilized automated sample transferring and SPE in 96-well plate format.

2. Experimental

2.1. Chemicals and reagents

MOR sulfate pentahydrate was from United States Pharmacopeia (Rockville, MD), M3G (purity 100%) and M6G dihydrate (purity 99%) were from Sigma Chemical Company (St. Louis, MO). Internal standards (IS) MOR-d_3 hydrochloride trihydrate (purity 99%) was also from Sigma. M3G-d_3 (purity 98%) and M6G-d_3 dihydrate (purity 99%) were from High Standard Products Corporation (Inglewood, CA). Acetonitrile, methanol, and water were of HPLC grade and were from Fisher Scientific (St. Louis, MO). Trifluoroacetic acid (TFA) was from Sigma. Control human sodium heparinized plasma was from Biochemed (Winchester, VA).

2.2. Calibration standards and quality control samples

Standards and quality control (QC) were made from two separate stock solutions of MOR, M6G and M3G. MOR stock solutions (1 mg ml^{-1}) were prepared by dissolving MOR sulfate pentahydrate in methanol. M6G or M3G stock solutions (0.1 mg ml^{-1}) were prepared by dissolving M6G dihydrate or M3G in methanol–water (20:80, v/v). Pooled calibration standards at concentrations of 0.50/1.00/10.0, 1.00/2.00/20.0, 2.50/5.00/50.0, 5.00/10.0/100, 10.0/20.0/200, 25.0/50.0/500, 40.0/80.0/800, and 50.0/100/1000 ng ml^{-1} for MOR/M6G/M3G were prepared in blank plasma. QCs at levels of 1.50/3.00/30.0, 15.0/30.0/300, and 37.5/75.0/750 ng ml^{-1} for MOR/M6G/M3G were prepared. Over the curve QCs were prepared at 100/200/2000 ng ml^{-1} for MOR/M6G/M3G. All standards and QCs were aliquoted and stored frozen at -20 °C.

2.3. LC-MS-MS

The LC-MS-MS system consisted of a Shimadzu HPLC system (Kyoto, Japan) and a PE Sciex API 3000 tandem mass spectrometer (Concord, Ontario, Canada) with (+) ESI. The analytical column, Betasil silica of 5 μm, 50×3.0 mm^2 I.D., was from Keystone Scientific (Bellefonte, PA). The injection volume was 5 μl; run time was 3.5 min; flow rate was 0.7 ml min^{-1}. Autosampler carry-over was determined by injecting the highest calibration standard then an extracted blank sample. No carry-over was observed. Without any column-regeneration, one column could be used for at least 1000 injections of the extracted samples.

Sensitivity of the multiple reaction mode (MRM) was optimized by testing with an infusion of 0.1 μg ml^{-1} each of the analytes in a mixture of acetonitrile and water (1:1, v/v). The Ionspray needle was maintained at 5 kV. The turbo gas temperature was 400 °C and the auxiliary gas flow was 8.0 l min^{-1}. Nebulizing gas, curtain gas, and collision gas flows were at instrument settings of 12, 8, and 4, respectively. The declustering potential and focusing potential were at 46 and

200 V, respectively. The mass spectrometer was operated under MRM mode with a collision energy of 77 V for MOR; and 45 V for both M6G and M3G. The transitions (precursor to product) monitored were m/z 286→152 for MOR, m/z 462→286 for M6G and M3G. The deuterated IS were monitored at m/z 289→152 for MOR-d_3, and m/z 465→289 for M6G-d_3 and M3G-d_3. The dwell time was 300 ms for the analytes and 100 ms for IS. Both quadrupoles were maintained at unit resolution.

Chromatograms were integrated using the Analyst version 1.1 software. A weighted 1/concentration^2 linear regression was used to generate calibration curves from standards and calculate the concentrations of QC samples.

2.4. Sample preparation

Samples were briefly vortex-mixed and centrifuged at 2000 rpm for 10 min on a Beckman Coulter J2-HS centrifuge (Fullerton, CA). Two hundred and fifty microliter were then transferred from vials into 1-ml 96-well deep well plates from Porvair Sciences (Shepperton, UK) by the Packard Multiprobe™ II robotic liquid handler (Meriden, CT) controlled by the WinPrep™ software. Between two consecutive pipetting, the Multiprobe needles were washed with water, 0.5% TFA in acetonitrile, and water. Carry-over of the Multiprobe needles was not observed. Twenty five microliter of IS spiking solution (50/100/1000 ng ml^{-1} for MOR-d_3/M6G-d_3/M3G-d_3 in water) were then added to all samples except blanks by the Multiprobe™ II. The sample plate was then brought to Tomtec Quadra™ 96-320 robot (Hamden, CT) and automated SPE was carried out. The 50 mg C_{18} SPE Versaplate™ cartridge plate (Varian Sample Preparations, Walnut Creek, CA) was first conditioned by 0.8 ml of methanol followed by 0.8 ml of 0.1% (v/v) TFA in water. Samples were mixed with 0.25 ml of 0.1% (v/v) TFA in water and then the mixture was loaded onto the cartridge plate. The plate was washed by 0.8 ml of 0.1% (v/v) TFA in water. After drying for ~3 min, samples were eluted using two portions of 0.4 ml of methanol–water (1:1, v/v) into another deep well collection plate. The collection plate was dried using a TurboVap™ 96 concentrator (Zymark, Hopkinton, MA) and reconstituted with 150 μl of acetonitrile–water–TFA (95:5:0.01, v/v/v) using the Tomtec robot. The advantage of using a reconstitution solution with elution strength weaker than the mobile phase has been discussed [15]. The plate was then heat-sealed with a Uniseal™ film (Whatman, Clifton, NJ) for injection onto LC-MS-MS system.

2.5. Validation of the LC-MS-MS method

The method was validated by three consecutive analytical curves on three separate days. Each calibration curve contained a single set of calibration standards and six replicates of QCs at each concentration level. One calibration curve also included over the curve QCs, which were diluted 5-fold with control blank plasma prior to analysis. Each curve also contained other test samples such as processing and storage stability samples. Calibration standards, QCs and other test samples were randomized through the curve. An extracted blank sample was always placed after the upper limit of quantitation standard to determine carry-over of the LC-MS-MS system. One curve contained more than 100 samples to simulate the length of clinical sample analysis.

The method specificity was evaluated by screening six lots of blank plasma. These lots were spiked with MOR/M6G/M3G at 0.00/0.00/0.00, 0.50/1.00/10.0, and 10.0/20.0/200 ng ml^{-1}. The spiked samples were extracted and analyzed to confirm lack of interferences and absence of lot-to-lot variation.

Analyte stability was tested by subjecting QCs through multiple freeze-thaw cycles, and on the bench at room temperature. Post-extraction analyte stability was also determined.

Recovery was determined by comparing the peak areas of the analytes extracted from plasma with those of post-extraction spiked plasma blanks at corresponding concentrations.

The method ruggedness was evaluated by injecting an extracted curve onto multiple LC-MS-MS instruments using analytical columns from different lots.

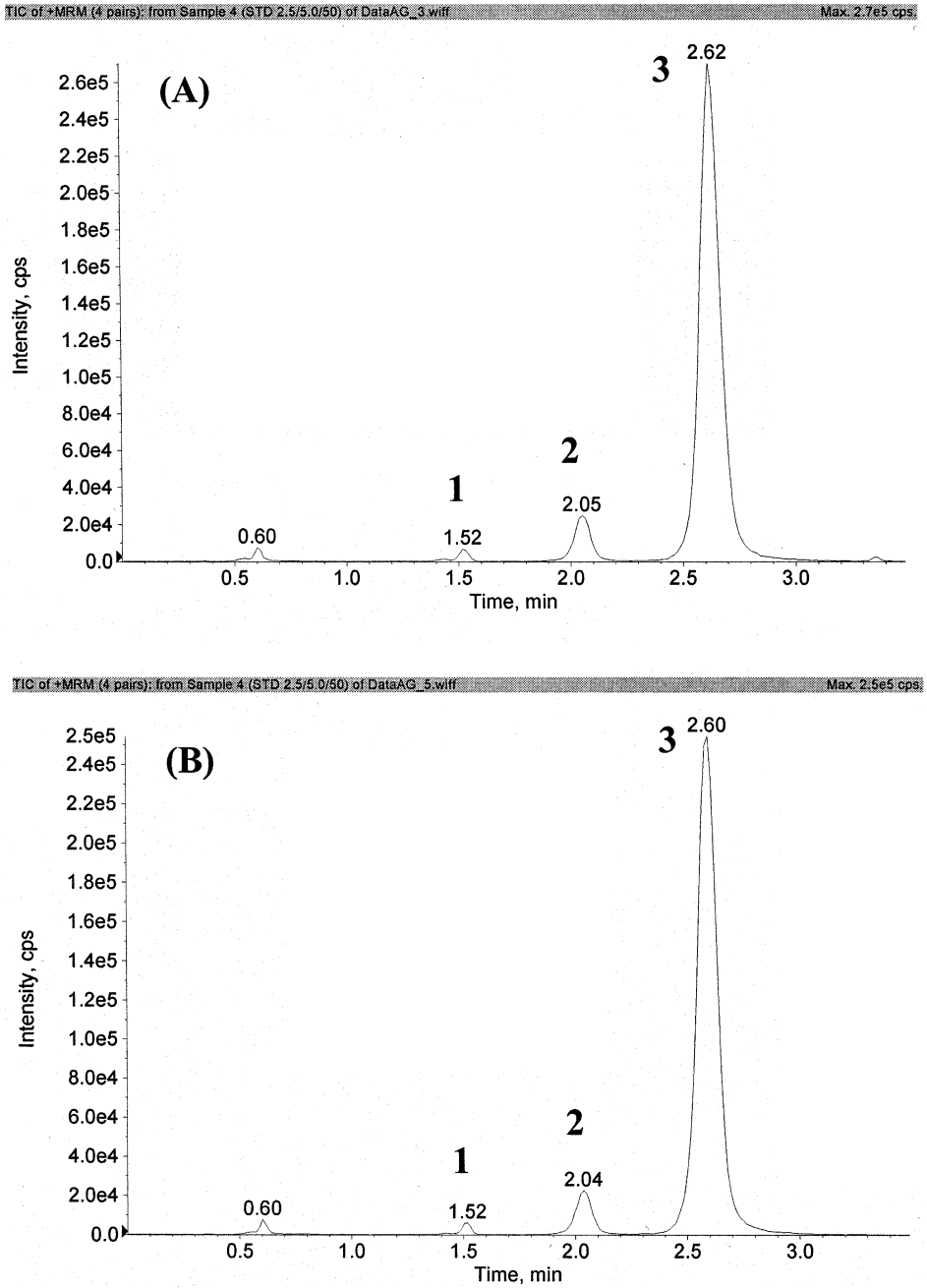

Fig. 1. Silica column stability: total ion current traces of MOR, M3G and M6G of the human plasma spiked with (1) 2.5 ng ml^{-1} MOR and 5 ng ml^{-1} MOR-d_3; (2) 5 ng ml^{-1} M6G and 10 ng ml^{-1} M6G-d_3; and (3) 50 ng ml^{-1} M3G and 100 ng ml^{-1} M3G-d_3. (A) Injection # 4; (B) Injection # 156.

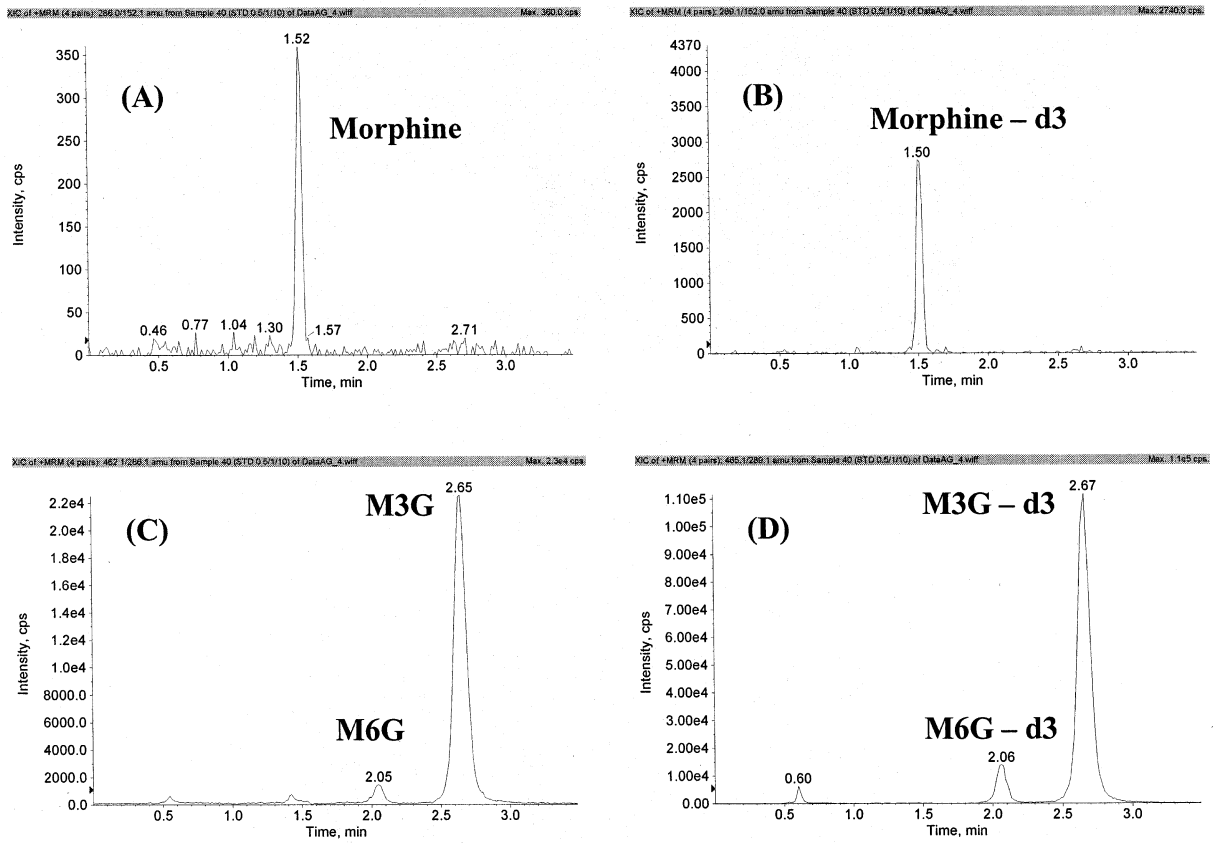

Fig. 2. Chromatogram of a blank plasma sample spiked with MOR/M6G/M3G at LLOQ (0.50/1.0/10 ng ml^{-1}) and IS. (A) MOR channel, 286 → 152; (B) MOR-d_3 (IS for MOR) channel, 289 → 152; (C) M6G and M3G channel, 462 → 286; (D) M6G-d_3 and M3G-d_3 (IS for M6G and M3G) channel, 465 → 289.

Table 1
Matrix lot-to-lot reproducibility

Matrix lot #		MOR		M6G		M3G	
	Theoretical concentration (ng ml^{-1})	0.500	10.0	1.00	20.0	10.0	200
1	Measured concentration (ng ml^{-1})	0.535	9.86	1.07	20.2	9.54	198
2		0.435	10.9	1.03	20.7	9.84	193
3		0.552	9.29	1.14	20.5	9.96	196
4		0.581	8.93	1.01	19.0	10.2	184
5		0.501	9.67	0.93	21.9	9.86	197
6		0.508	9.75	1.01	20.1	10.6	199
	Mean	0.519	9.73	1.03	20.4	10.0	195
	RSD (%)	8.9	6.3	6.2	4.2	3.3	2.6
	RE (%)	+3.8	−2.7	+3.0	+2.0	0.0	−2.5

3. Results and discussion

3.1. LC-MS-MS

The selection of silica column and aqueous-organic mobile phase for resolving MOR, M6G and M3G was based on our previous experience [8]. The conjugated metabolites could fragment to MOR in the LC-MS interface and be falsely detected as MOR if chromatographic separation was not achieved [8,16]. Bare silica columns operated with aqueous-organic mobile phases are viable means of analyzing polar compounds in biological fluid [17,18]. The previously described chromatographic condition was further improved by replacing 1% (v/v) formic acid with 0.01% (v/v) TFA in the mobile phase. The water content was decreased from 30% with the formic acid mobile phase to 9% with the TFA mobile phase. The chromatographic separation between M6G and M3G was improved. Even though TFA was reported to suppress electrospray signals due to its ion-pairing in the gas phase with the analyte ions [19], the gain of sensitivity by using higher organic content was so large that the overall sensitivity was still enhanced. The increase on sensitivity by going to TFA mobile phase on silica column has been observed for fentanyl, a potent synthetic analgesic [20]. The silica column demonstrated excellent stability as shown in Fig. 1, indicated by very stable retention time and unchanged peak shape for all three analytes.

3.2. Automation

The automation strategy involved in this method is to separate the sample transfer step from the

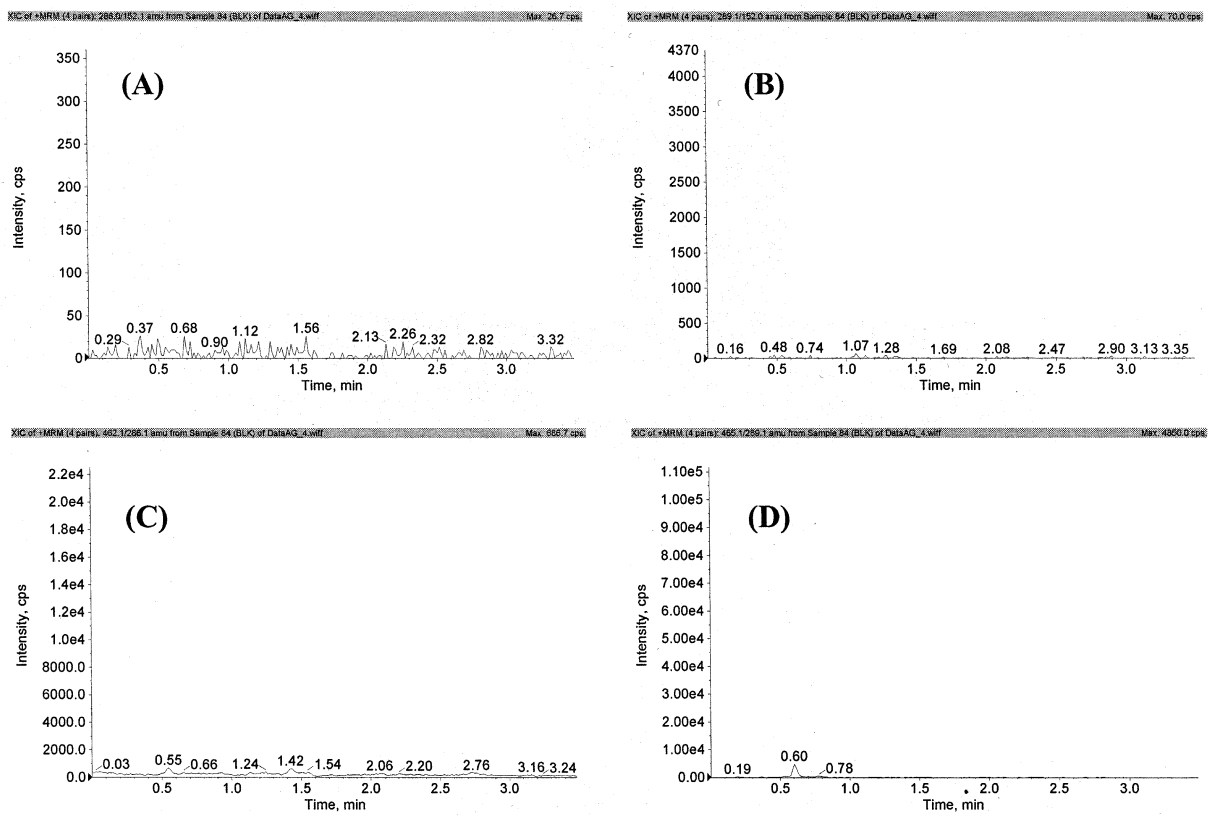

Fig. 3. Chromatogram of a blank plasma sample. (A) MOR channel, 286 → 152; (B) MOR-d_3 (IS for MOR) channel, 289 → 152; (C) M6G and M3G channel, 462 → 286; (D) M6G-d_3 and M3G-d_3 (IS for M6G and M3G) channel, 465 → 289.

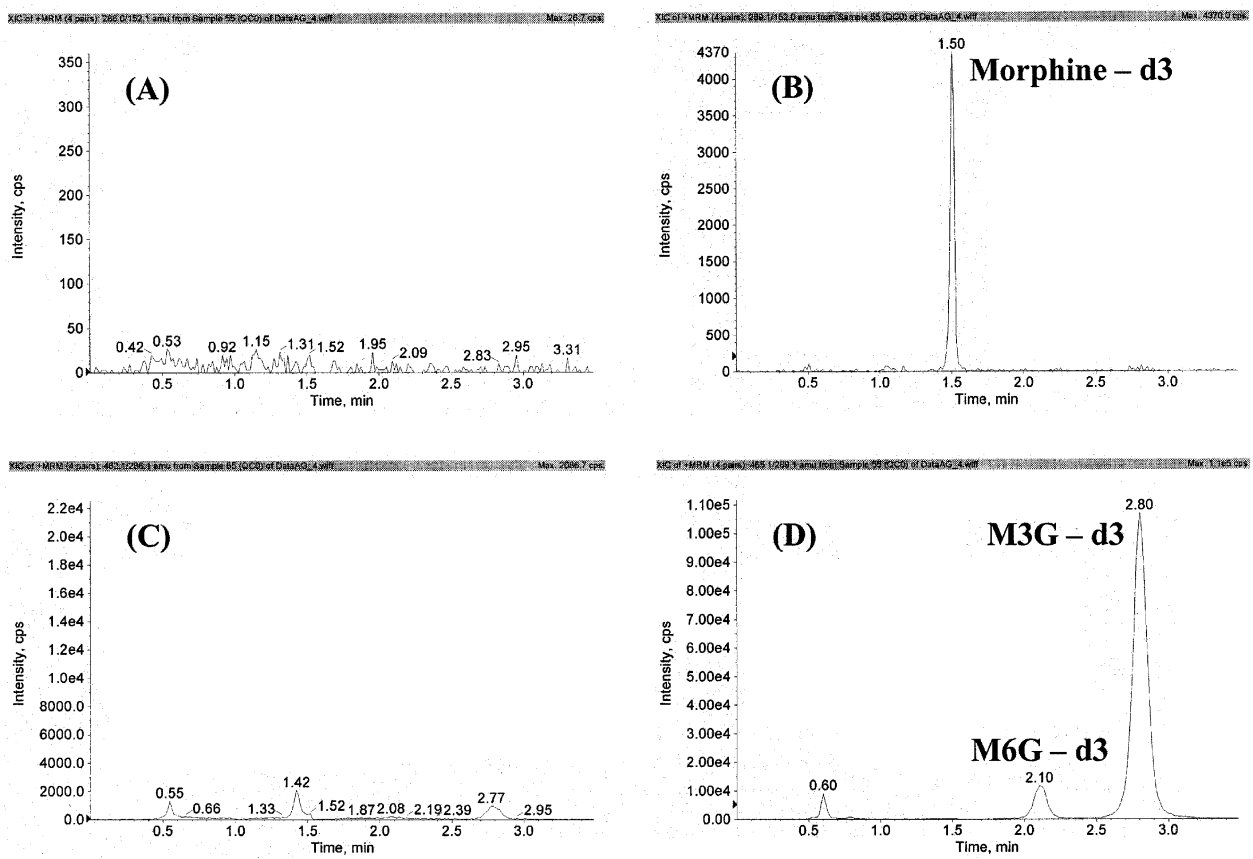

Fig. 4. Chromatogram of a blank plasma sample spiked with only IS. (A) MOR channel, 286→152; (B) MOR-d_3 (IS for MOR) channel, 289→152; (C) M6G and M3G channel, 462→286; (D) M6G-d_3 and M3G-d_3 (IS for M6G and M3G) channel, 465→289.

extraction step. The use of a single robotic liquid handler with either 4 or 8 tips (i.e. Packard Multiprobe™ II) was ideal for the sample transfer. It should be pointed out that unlike handling EDTA plasma samples as we described in an earlier publication [20], transferring heparin plasma with the Multiprobe required extra attention from the chemist. Occasional needle-tip blockage due to fibrogen in heparin plasma did occur but subsequent pipetting was not adversely affected due to extensive wash steps between consecutive samples. These few samples were transferred manually by the chemist into the 96-well plate. Despite this drawback, utilization of Multiprobe still led to excellent pipetting accuracy and precision, as demonstrated in Section 3.3. The good precision was reflected by the very low relative standard deviation (RSD) values for the calibration standards and QCs. The good accuracy was demonstrated by the very low RE values for QCs (37.5 and 100 ng ml^{-1}) which were diluted 5-fold by the Multiprobe.

Packard Multiprobe™ II has the flexibility of pipetting samples from tubes to 96 well plate or to another set of tubes. Due to limited numbers of tips, the Multiprobe™ II needs to carry out SPE analysis in a serial fashion. On the other hand, the Tomtec Quadra™ 96 workstation is equipped with 96 tips and is capable of extracting 96 samples simultaneously. In the current method, the Multiprobe™ II was programmed to aliquot samples from individual tubes to 96-well deep well plates and to add the IS. The plate was then brought to Quadra™ 96 for the SPE sample cleanup. This separation of the two steps not only maximized the ability of Multiprobe™ II's ability to accurately

Table 2
Precision and accuracy of calibration standards ($n = 3$)

MOR (ng ml^{-1})	0.500	1.00	2.50	5.00	10.0	25.0	40.0	50.0	slope	r^2
Mean	0.503	1.01	2.40	4.88	10.1	24.8	41.7	50.3	0.253	0.9992
RSD (%)	0.5	1.3	2.3	1.1	2.6	4.0	4.3	2.5	0.8	0.03
RE (%)	+0.6	+1.0	−4.0	−2.4	+1.0	−0.8	+4.3	+0.6		
M6G (ng ml^{-1})	1.00	2.00	5.00	10.0	20.0	50.0	80.0	100	slope	r^2
Mean	1.00	2.01	4.93	10.1	19.9	49.2	80.8	100	0.0798	0.9997
RSD (%)	1.7	2.2	1.3	2.8	3.6	1.8	0.7	1.6	6.3	0.02
RE (%)	0.0	+0.5	−1.4	+1.0	−0.5	−1.6	+1.0	0.0		
M3G (ng ml^{-1})	10.0	20.0	50.0	100	200	500	800	1000	slope	r^2
Mean	9.86	20.2	52.1	101	202	486	787	988	0.0181	0.9993
RSD (%)	1.2	2.1	3.2	3.6	2.3	2.7	1.5	2.3	5.1	0.03
RE (%)	−1.4	+1.0	+4.2	+1.0	+1.0	−2.8	−1.6	−1.2		

and flexibly transfer samples and IS, but also fully utilized the Quadra™ 96's parallel processing capability. The sample preparation throughput for 96 samples improved from ≈ 5.5 h for manual operation to about 3 h for using the combination of Multiprobe™ II and Quadra™ 96. This comparison result is similar to those previously reported [21]. The major time saving step was the extraction step (~ 30 min for automated extraction vs. ~ 120 min for manual extraction).

3.3. Validation results

Six lots of blank control plasma were tested for matrix interference. The regions of the analytes and their deuterated IS peaks were free from interferences. When the samples were spiked with the analytes at their low limit of quantitation (LLOQ), which was 0.500/1.00/10.0 ng ml^{-1} for MOR/M6G/M3G, the RSDs and relative errors (REs) were 8.9/6.2/3.3% and +3.8/+3.0/0.0% for MOR/M6G/M3G, respectively (Table 1). For the samples spiked with 10.0/20.0/200 ng ml^{-1} of MOR/M6G/M3G, the RSDs and REs were 6.3/4.2/2.6% and −2,7/+2.0/−2.5%, respectively. These tight RSD and RE values indicate no significant lot-to-lot variation in matrix effects.

Representative chromatograms of a LLOQ, a blank matrix, and a control zero (blank plasma spiked with IS only) are shown in Figs. 2–4. The signal to noise ratio for MOR is about 35 at 0.5 ng ml^{-1}, which is about 5-fold sensitivity improvement over the previous method [8]. The sample volume has also decreased from 1 to 0.25 ml in the current method.

Recoveries were determined by comparing the peak areas of extracted QC samples with peak areas of post-extraction spiked plasma blanks at corresponding concentrations. The mean recoveries of MOR/M6G/M3G were at least 43/72/71% for the three QC concentrations. The overall mean recoveries were 48/79/74% for MOR/M6G/M3G. The recoveries for M6G and M3G were comparable with previous result [8], but the MOR recovery was about 40% lower. The lower recovery for MOR was due to lack of means of preventing cartridges from going dry in the 96-well format when vacuum was used. For the current method, the lower recovery for MOR was not an issue since deuterated IS was employed and much improved sensitivity was observed.

Calibration curve parameters and data are listed in Table 2. The correlation coefficients of the three validation curves were all > 0.998. 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^{-1} for MOR, 1–100 ng ml^{-1} for M6G, and 10–1000 ng ml^{-1} for M3G, using weighted (1/concentration^2) least-square linear regression. At the LLOQ, the RSD ($n = 6$) of the measured concentration was 5.1/8.2/1.5% for MOR/M6G/M3G. The RE of the mean of the measured concentrations were −3.4/−0.2/0.0% for MOR/M6G/M3G.

The precision and accuracy data for QC samples

Table 3
Precision and accuracy of QC samples

	Intraday ($n = 6$)					Interday ($n = 18$)		
MOR (ng ml^{-1})	1.50	15.0	37.5	37.5[a]	100[a]	1.50	15.0	37.5
Mean	1.56	16.0	40.0	40.5	107	1.60	15.5	38.4
RSD (%)	7.7	2.4	2.9	2.9	4.7	8.3	4.4	5.6
RE (%)	+4.0	+6.7	+6.7	+8.0	+7.0	+6.7	+3.3	+2.4
M6G (ng ml^{-1})	3.00	30.0	75.0	75.0[a]	200[a]	3.00	30.0	75.0
Mean	2.95	30.3	75.8	78.1	203	2.93	30.1	75.6
RSD (%)	3.3	1.8	0.4	3.7	2.7	4.5	2.6	1.6
RE (%)	−1.7	+1.0	+1.1	+4.1	+1.5	−2.3	+0.3	+0.8
M3G (ng ml^{-1})	30.0	300	750	750[a]	2000[a]	30.0	300	750
Mean	30.6	300	710	788	2020	30.6	299	719
RSD (%)	2.3	1.8	2.0	3.9	2.5	2.0	2.1	2.4
RE (%)	+2.0	0.0	−5.3	+5.1	+1.0	+2.0	−0.3	−4.1

[a] Samples were diluted five fold with blank plasma prior to analysis.

Table 4
Stability of the samples

	Concentration (ng ml^{-1})								
	MOR			M6G			M3G		
	1.50	15.0	37.5	3.00	30.0	75.0	30.0	300	750
3 Freeze/thaw cycles ($n = 3$)									
Mean	1.55	15.7	38.0	2.86	31.4	76.3	31.4	300	725
RSD (%)	7.5	3.1	3.1	1.1	1.9	2.5	1.6	2.3	0.6
RE (%)	+3.3	+4.7	+1.3	−4.7	+4.7	+1.7	+4.7	0.0	−3.3
24 h bench-top ($n = 3$)									
Mean	1.70	15.2	36.1	2.96	31.3	76.2	30.9	310	725
RSD (%)	12.6	2.3	3.2	5.9	1.9	1.7	1.0	0.9	2.0
RE (%)	+13.3	+1.3	−3.7	−1.3	+4.3	+1.6	+3.0	+3.3	−3.3
24 h extract ($n = 6$)									
Mean	1.54	15.2	38.5	3.10	30.4	75.5	30.3	295	694
RSD (%)	7.4	5.6	2.4	6.3	1.9	2.8	2.1	1.5	0.6
RE (%)	+2.7	+1.2	+2.7	+3.3	+1.3	+0.6	+0.9	−1.8	−7.4

are summarized in Table 3. The data show that this method is consistent and reliable with low RSDs and REs values.

The stability tests were designed to cover the anticipated conditions that the clinical samples may experience. Stabilities of sample processing (freeze-thaw and bench-top), and chromatography (extracts) were tested and established. The results are summarized in Table 4. Three freeze/thaw cycles and ambient temperature storage of the QC samples for up to 24 h prior to analysis, appeared to have little effect on the quantitation. QC samples stored in a freezer at −20 °C remained stable through the course of the validation. Extracted calibration standards and QC samples were allowed to stand at ambient temperature for 24 h prior to injection. No effect on quantitation of the calibration standards or QC samples was observed. The method robustness was demonstrated by using multiple analytical

columns and LC-MS-MS instruments. This method has been successfully used to analyze samples from clinical trials. As already described in the literature [8], the clinical sample concentrations were within the current curve ranges for MOR (0.5–50 ng ml^{-1}), M6G (1.0–100 ng ml^{-1}) and M3G (10–1000 ng ml^{-1}).

4. Conclusion

A sensitive, reliable and highly automated LC-MS-MS method for the measurement of MOR, and its metabolites M6G and M3G, in human sodium heparin plasma has been successfully developed and validated. Deuterated IS for each analyte minimized the potential bias caused by inconsistent matrix effects and improved the method ruggedness. A silica column and an aqueous-organic mobile phase were used to achieve chromatographic resolution of the three analytes, which is important for avoiding artificial over-estimation of MOR due to in-source deconjugation of M3G. The LLOQ is 0.5 ng ml^{-1} for MOR and 1 ng ml^{-1} for M6G by using only 0.25 ml plasma. The highly automated nature of the method significantly improved the sample analysis throughput.

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