

HPLC-MS/MS Based Time Course Analysis of Morphine and Morphine-6-Glucuronide in ICU Patients

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Abstract

Morphine is a widely-used opioid analgesic to treat post-operative pain in the intensive care unit. For the quantification of morphine and its metabolite Glucuronide (M6G) concentrations a sensitive and specific liquid chromatography–tandem mass spectrometry (LC-MS/MS) method was developed and validated according to Food and Drug Administration (FDA) guidelines. Plasma samples were extracted with solid-phase extraction and substituted with deuterated morphine and M6G as internal standards. Separation was performed by gradient elution using UPLC-like system and analyzed by MS/MS consisting of an electrospray ionization source. The lower limit of quantification was 500 “pg/ml” for morphine and 50 “pg/ml” for M6G. Intra- and interassay precision and accuracy did not exceed $\pm 15\%$. The method was applied to a clinical study during intensive care treatment of patients after coronary artery bypass grafting and can serve for further pharmacokinetic studies.

Keywords: Morphine; Morphine-6-glucuronide; High performance liquid chromatography; Tandem mass spectrometry; Pharmacokinetic; Intensive care

Introduction

Morphine is an opioid analgesic used for the treatment of moderate to severe pain. It is also commonly used to treat post-operative pain in the intensive care unit (ICU) [1]. Morphine is metabolized by conjugation to morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G) [2]. Whereas, the main metabolite M3G, has little or no analgesic effect, M6G has been shown to be very effective and is believed likely to contribute significantly to the overall effectiveness of morphine. Impairments of the hepatic and renal function [3] may alter morphine elimination and conjugation, especially in critically ill and ICU patients [4]. To date, there are only a few reports on quantification of morphine and its metabolites in ICU patients [5]. Nevertheless, morphine has been widely investigated in terms of efficacy and side effects in other patient groups. In critical ill patients, metabolism of morphine through UDP-glucuronosyltransferase 2B7 to M6G and renal excretion of morphine and M6G may be affected with major surgery such as cardiac surgery with cardiopulmonary bypass or critical illness like septic shock or multiple organ failure [5].

The quantification of morphine and its metabolites may present several analytical problems in biological fluids. HPLC-MS/MS is the method of choice for simultaneous analysis of morphine and M6G in human serum reviewed by Bosch et al. [6] and was used for this setting. Our investigations were intended to provide a robust and validated, according to Food and Drug Administration (FDA) guidelines [7], HPLC-MS/MS protocol for determination of morphine and M6G in human plasma and give example for clinical use in intensive care patients. The method was applied to a clinical study in ICU patients after cardiac surgery with cardiopulmonary bypass. Pain treatment were performed via patient controlled analgesia (PCA) and plasma samples were drawn frequently. Deuterated morphine and M6G were used as internal standards to improve the prediction and accuracy of the method as well as the robustness of the quantification against matrix effect. Tandem mass spectrometry was used for specific and reliable detection and quantification of the analytes.

Materials and Methods

Drugs and chemicals

Morphine, M6G and the internal standards morphine-D6 and M6G-D3 (Figure 1) were purchased from LGC-Standards (Wesel, Germany). Ammoniac (30%), Methanol (LC-MS grade), ultra LC-MS water and formic acid were purchased from Roth (Karlsruhe, Germany). Drug free human plasma was obtained from Recipe Chemicals (München, Germany).

Standard solution and calibration standards

Stock solutions of morphine, morphine-D6, M6G and M6G-D3 were 1000 $\mu\text{g/ml}$, and calibration standards were prepared with methanol and stored at -20°C . Quality control (QC) samples were prepared to drug free plasma and blank study plasma samples (zero samples) by adding 50 μl standards to 450 μl drug free plasma. The calibration samples for the determination of drug concentrations (morphine 0.5–100 ng/ml, M6G 0.05–10 ng/ml) were prepared in the same way using drug free plasma.

Extraction procedure

For the assay samples were supplied together with IS (50 μl of 500 ng/ml deuterated internal standard) and 1 ml formic acid 0.1% after centrifugation at 5000 rpm for 1 min to OASIS MCX solid phase extraction cartridges (Waters, USA), which had been conditioned with 2 ml of methanol followed by 2 ml formic acid 0.1%. The cartridges were rinsed twice with 1 ml formic acid 0.1% under a slight vacuum. For descaling the residual water, the cartridges were centrifuged again for 1 minute at 5000 x g. The analytes were then eluted with 1000 μl

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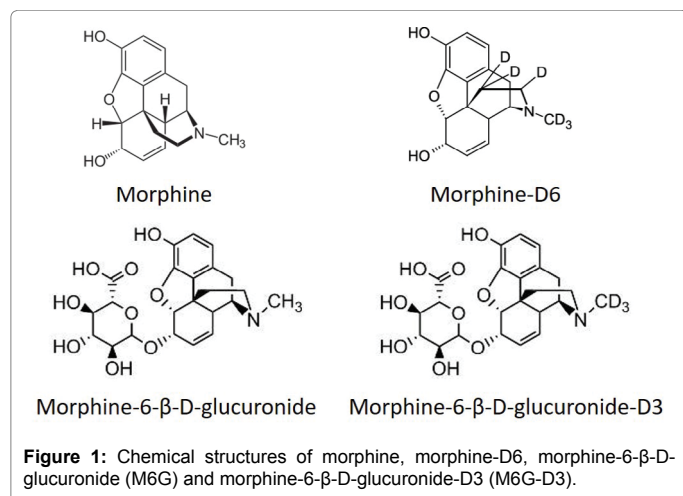


Figure 1: Chemical structures of morphine, morphine-D6, morphine-6-β-D-glucuronide (M6G) and morphine-6-β-D-glucuronide-D3 (M6G-D3).

methanol, containing 5% ammoniac. After evaporating to dryness, the extracts were solved in 150 µl formic acid 0.1%.

Equipment

The extracted samples were analyzed with HPLC and tandem mass spectrometry. Chromatographic separation was carried out with the Waters Alliance HPLC system (Waters, Eschborn, Germany). The system was upgraded and modified by Fischer Analytics (Bingen, Germany) to allow UPLC-like pressures of more than 400 bars. For chromatographic separation, a Kinetex (Biphenyl, 50 × 2.1 mm, 1.7 µm, 100 Å) analytical column (Phenomenex, Aschaffenburg, Germany) protected by a HPLC Guard Cartridge system (Biphenyl Security Guard, Phenomenex) was used. The analytes were detected with a Waters Quattro Micro tandem mass spectrometer equipped with an electrospray ionization interface (ESI). Data were collected and analyzed with MassLynx™ V4.1 software.

HPLC conditions

The chromatographic separation of the analytes was accomplished by gradient elution. The separation was started with mobile phase consisting of 5% methanol, containing 0.1% formic acid and 95% formic acid 0.1% (5:95, v/v). After 0.3 minutes the fraction of methanol was raised to 80% and held constant for 2.5 minutes (80:20, v/v). The system was re-equilibrated with starting conditions (5:95, v/v) for the next 4 minutes. Total chromatographic time was 8 minutes. Changes in mobile phase composition were achieved using step gradient. The mobile phase flow was kept at 400 µl/min and 20 µl of the extracted sample was injected for. With a column temperature of +30°C, the retention times for morphine and morphine-D6 were about 1.88 respectively 1.96 minutes for M6G and M6G-D3.

Mass spectrometry conditions

Ionization and analysis were accomplished in positive electrospray mode with multiple-reactions monitoring (MRM). The following settings for mass spectrometry were used morphine/M6G: ion source temperature was set at 120°C, the capillary and cone voltages were set to 3.8 kV and 45 V/65 V, respectively. The cone gas flow was 50 l/min. Desolvation gas temperature was 500°C and the flow was set to 900 l/min. Nitrogen was used as the desolvation and cone gas, and argon was used as the collision gas. The product ions were 286 → 165 for morphine, 292 → 165 for morphine-D6, 462 → 286 for M6G and 465 → 289 for M6G-D3. Dwell time was 200 ms for all ion transitions monitored.

Method validation

The method was validated according to the Food and Drug Administration (FDA) guidelines on recovery, linearity, precision, accuracy, selectivity, and specificity [7].

Loss of morphine and M6G during extraction recovery: The efficiency of the extraction procedure of morphine, M6G and internal standards from human plasma was analyzed in triplicates at concentrations of 0.5, 10 and 100 ng/ml for morphine and 0.05, 1, 10 ng/ml for M6G in human plasma. Recovery was calculated by comparison of the results obtained for samples extracted according to the analytical procedure of total plasma concentration with those obtained for the standard solutions added to the blank plasma extracts, corresponding to 100% recovery.

Linearity: Assay linearity was evaluated on 3 consecutive days by constructing freshly prepared plasma calibration samples over the concentration range of 0.5 to 100 ng/ml (morphine) and 0.05 to 10 ng/ml (M6G). The calibration curves were generated with Target Lynx software (v. 4.1; Waters) using linear regression.

The lower limit of quantification (LLOQ) for morphine and M6G was defined as the lowest standard on the calibration curves with a peak response at least five times that of the blank response (S/N ratio) and with precision of 20% and accuracy of 80-120%.

Precision and accuracy: Intraday accuracy and precision were evaluated by analysis of 3 calibration standards per range and 5 replicates of each QC sample concentration in one day. For the assessment of the interday accuracy and precision of the method, sets consisting of calibration standards and 3 different concentrations of QC samples (0.5, 10, 100 ng/ml for morphine and 0.05, 1, 10 ng/ml for M6G) were run on 4 to 16 days. Each set contained 6 calibration standards and 2 to 5 replicates were analyzed. The precision was determined as the intra-assay and inter-assay relative standard deviations (%RSD) of the determined concentrations: %RSD=100·SD/M, where M is the mean and SD is the standard deviation. Accuracy was expressed as the relative error from nominal concentration: %RE=100 (experimentally obtained concentration–nominal concentration)/(nominal concentration). The acceptance criteria for accuracy were ± 15% deviation from nominal values and for precision %RSD less than 15%.

Selectivity and specificity: Test for selectivity was carried out in six lots of blank patient plasma. In the first set, the blank human plasma was directly injected after extraction (without analyte and IS), while the other set was spiked with IS and LLOQ concentration of morphine or M6G. The acceptance criterion requires that at least 90% of selectivity samples should be free from any interference at the retention time of analyte and IS. The specificity of the assay was evaluated by comparing chromatograms of a blank plasma sample, a blank plasma sample spiked with 0.5 ng morphine or 0.05 ng M6G and IS; and a patient's plasma sample containing morphine and M6G.

Stability

Short-term stability, post-processing stability, stability after three freeze–thaw cycles, and stock solution stability were evaluated according to the analytical procedure of plasma concentration. For this purpose, 3QC samples per drug range were prepared: 0.5, 10, 100 ng/ml for morphine and 0.05, 1, 10 ng/ml for M6G. The samples were maintained at room temperature (+20°C) for 24 h for the analysis of short-term stability. Post-processing stability was evaluated by maintaining the samples in the autoinjector at 25 °C for six days, followed by injection into the chromatographic system. For the evaluation of stability after

three freeze–thaw cycles, the QC samples were frozen at -75°C for 24 h. After this period, the samples were again thawed and frozen for 24 h and this process was repeated until the third thawing cycle when the samples were extracted and analyzed. The stability of stock solutions was evaluated after maintaining them at room temperature ($+20^{\circ}\text{C}$) for 12 h. Long term stability was tested after storing the QC-samples at -75°C for 12 months, after which the samples were analyzed. The results were compared to those obtained for freshly prepared samples and are expressed as relative error (%RE). The solutions were considered stable if the deviation from nominal value was within $\pm 15.0\%$.

Application to clinical study

The method was applied to investigate the concentration time courses of morphine and its metabolite M6G during intensive care treatment of 25 patients after cardiac surgery with cardiopulmonary bypass. These patients participated in a clinical study registered with the ClinicalTrials.gov and EudraCT databases (identifiers NCT02483221 and 2014-004088-19, respectively). Anesthesia was induced and maintained with target controlled infusions (TCI) of propofol (Disoprivan[®] 2%, AstraZeneca, Wedel, Germany) using the pharmacokinetic model of Marsh et al. [8] and targeting plasma concentrations between 2.5 and 4 $\mu\text{g}/\text{ml}$. After the end of the surgery, the patients were transferred to the ICU where the propofol infusion was continued for further 2-3 h until weaning from mechanical ventilation with an infusion rate of 2-3 $\text{mg}/\text{kg}/\text{h}$. Post-operative pain therapy was performed with morphine PCA. 17 blood samples for the pharmacokinetic measurements were drawn at baseline and 1, 3, 5, 7, 10, 30, 60, 120 minutes and two hourly after starting the PCA. To determine the arterial morphine and M6G concentrations, 4 ml blood per sample were drawn from an artery line (S-Monovette[®] Kalium EDTA, Sarstedt, Nürnberg, Germany), after 1 ml blood had been drawn and discarded. After each sample collection, the intra-arterial catheter was flushed with 2 ml of heparinized NaCl-solution. The samples were kept on ice and plasma was separated within 15 minutes and stored at -75°C until analysis. Samples with concentrations above the linear calibration range ($>100 \text{ ng}/\text{ml}$ for morphine and $>10 \text{ ng}/\text{ml}$ for M6G) were diluted to fit within the range and were reanalyzed.

Results and Discussion

The aim of our investigation was to develop and validate an analytical method to measure concentrations of morphine and its metabolite M6G from human plasma samples, and the application of this method to a clinical study where morphine was used for pain therapy after cardiac surgery with cardiopulmonary bypass. To our knowledge there are no validated investigations in intensive care patients, which describe the analysis of morphine and M6G plasma concentrations in humans.

The previously published methods were modified also by using HPLC-system upgraded to be used in UPLC-like pressures to further improve the analytical performance of the system. Furthermore, deuterated morphine and M6G was utilized as internal standards to increase the robustness against possible matrix effects and to increase the accuracy and precision of the method. The method was validated according to the Food and Drug Administration (FDA) guidelines [7].

Recovery of morphine and M6G

There was no observed inconsistency in the recovery of morphine and M6G. The relative extraction recoveries of morphine and M6G were determined at 3 QC samples per drug: 0.5, 10, 100 ng/ml with 98.9, 103.0 and 100.4% for morphine and 0.05, 1, 10 ng/ml with 96.7, 99.4 and 99.1% for M6G.

Chromatography and mass spectrometry

The best chromatographic separation was obtained with a mobile phase containing a low acid concentration and high percentage of organic modifier. To get an ESI compatible mobile phase the analytes were injected with 0.1% formic acid. The fraction of acetonitrile was raised to 80% during the separation process. Ionic strength and pH were kept stable by adding formic acid to all eluents. A flow rate of 0.4 ml/min were used during the whole run without any loss in quality of chromatographic separation.

Analytical conditions for mass spectrometry were tested to obtain a high intensity of $[\text{M}+\text{H}]^+$ ions of the analytes. The positive product ion mass spectra showed high abundances of fragment ions. The product ion for morphine was 286, for M6G 462. The precursor \rightarrow product ion transformations of m/z 286 \rightarrow 165 (Figure 2A) and m/z 462 \rightarrow 286 (Figure 2B) were used for MRMs for morphine and M6G, respectively. No interference from endogenous substances in the blank plasma lots or from other metabolites was observed in the retention time of morphine and M6G.

Linearity and limits of detection and quantification

Linear calibration curves were identified for morphine and M6G concentrations within the range from 0.5 to 100 ng/ml ($y=61.9x$; $r^2=0.995$) and from 0.05 to 10 ng/ml ($y=2.03x+0.018$; $r^2=0.999$). The Limit of Detection (LOD) for morphine was 10 pg with a S/N ratio of 21.91 and 5 pg with a S/N ratio of 32.13 for M6G. The lower limit of quantification was set at 0.5 ng/ml for morphine and 0.05 ng/ml for M6G. Representative chromatograms of plasma spiked with the LOD concentrations of morphine and M6G is presented in Figure 3.

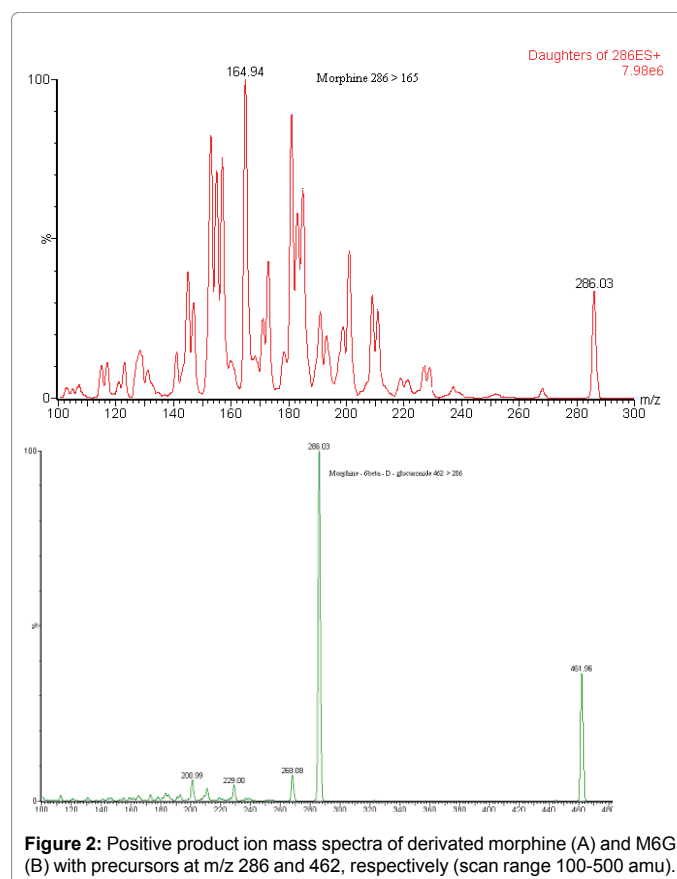
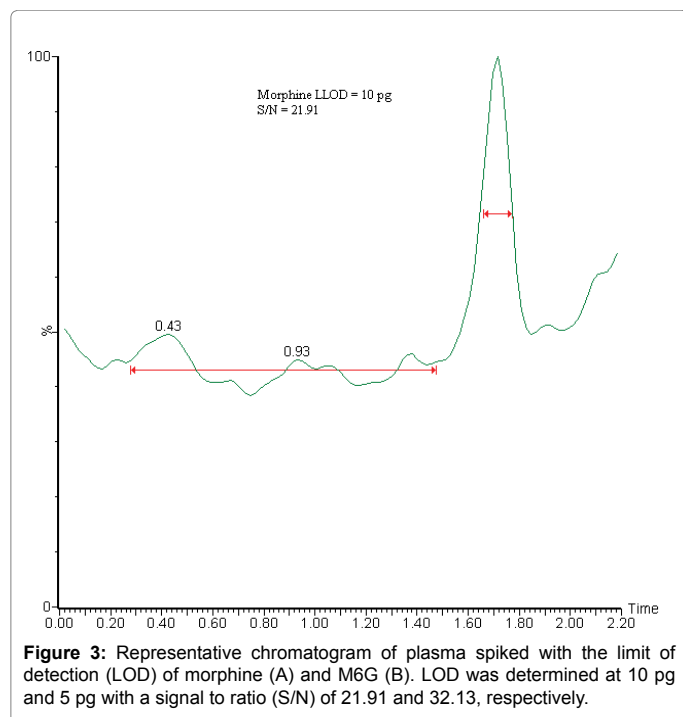


Figure 2: Positive product ion mass spectra of derived morphine (A) and M6G (B) with precursors at m/z 286 and 462, respectively (scan range 100-500 amu).



Precision and accuracy

The validation data regarding intraday and interday precision and accuracy are summarized in Table 1. The measurement of morphine and M6G by our method showed good intraday and interday precision and accuracy. Compared to previous studies, the utilization of deuterated morphine and deuterated M6G improved the intra- and interday accuracy and precision.

Selectivity and specificity

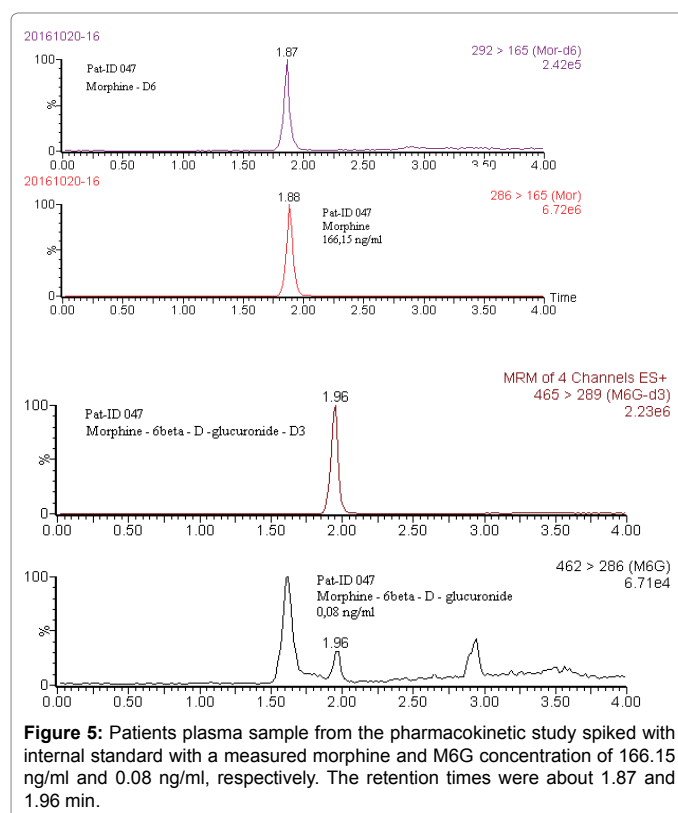
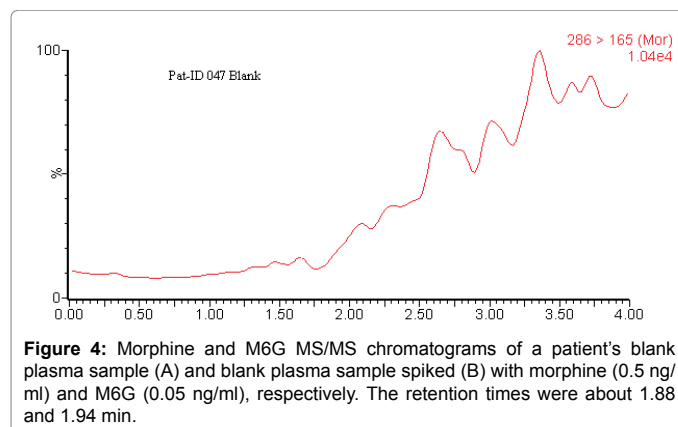
Specificity was evaluated by comparing chromatograms of blank patient plasma with corresponding drug-free plasma sample spiked with morphine or M6G and IS and with a blank patient samples from the clinical study. None of the six blank plasma lots caused a significant interference at the retention times of the analytes or internal standards. The signal response ratios (blank interference/LLOQ standard) were within the acceptable criteria for all studied compounds (Figures 4 and 5). The matrix effect at QC concentration level of 0.5 ng/ml (n=5) was 0.51 ± 0.04 (%RSD 8.30) for morphine and QC concentration level of 0.05 ng/ml (n=5) was 0.06 ± 0.005 (%RSD 8.49) for M6G indicating that no co-eluting substance influenced the ionization of the analytes.

Stability

No significant degradation of the analytes were observed during the short-term (24-hour) storage at room temperature, three freeze-thaw cycles, or long-term (>2-month) storage at -75°C , suggesting that no stability related problems are to be expected during routine analysis (Table 2). The degradation was less than 15% for all compounds. Also, the stock solutions showed no significant degradation after short term (12 hour) storage at room temperature.

Pharmacokinetic application

This method was utilized in a clinical trial to investigate the time course of morphine and its active metabolite morphine-6-glucuronide (M6G) in patients after cardiac surgery with cardiopulmonary bypass. Following a total intravenous anesthesia with propofol and



intraoperative analgesia with sufentanil a morphine based patient controlled analgesia (PCA) was applied for post-operative pain therapy. During the post-operative phase at the ICU the morphine and M6G plasma concentrations were measured in 17 blood samples of 6 patients over a time period of up to 18 h between arrival at the ICU and the following post-operative day. The morphine concentrations varied between 0.77 and 324.4 ng/ml, the M6G concentrations varied between 0.18 and 20.86 ng/ml. All samples were above the LLOQs and shows a time course of morphine and M6G concentration in a representative patient, respectively.

Conclusion

Our investigation demonstrates a sensitive and specific analytical method for determining the concentration of morphine and M6G. The method was used successfully to measure morphine and M6G plasma

	Nominal concentration (ng/ml)	Intraday				Interday			
		Mean ± SD	%RSD	%RE	n	Mean ± SD	%RSD	%RE	n
Morphine	0.5	0.51 ± 0.04	8.3	2.2	5	0.52 ± 0.04	7.74	4.08	20
	10	10.42 ± 0.52	4.96	4.2	5	10.2 ± 0.66	6.52	1.64	11
	100	99.30 ± 1.51	1.52	-0.7	5	97.8 ± 1.85	1.9	-2.15	11
Morphine-6-glucuronide	0.05	0.06 ± 0.005	8.49	11.2	5	0.05 ± 0.006	10.64	5.64	11
	1	1.03 ± 0.054	5.21	2.8	5	0.99 ± 0.067	6.84	-1.43	11
	10	9.51 ± 0.27	2.83	-4.9	5	9.7 ± 0.24	2.51	-3.47	11

%RSD: Relative standard deviation; %RE: Relative error from nominal concentration

Table 1: Accuracy and precision of the analytical method for the concentration measurement of morphine and morphine-6-glucuronide in human plasma.

	Mean ± SD (% RE)					
	Morphine			Morphine-6-glucuronide		
	0.5 ng/ml	10 ng/ml	100 ng/ml	0.05 ng/ml	1 ng/ml	1 ng/ml
Freeze and thaw stability (n=5)	0.5 ± 0.04 (-3.88)	9.8 ± 0.64 (-2.36)	96.8 ± 1.12 (-3.22)	0.049 ± 0.01 (-1.2)	0.96 ± 0.06 (-3.58)	9.8 ± 0.12 (-2.12)
Short-term stability ^a (n=4)	0.50 ± 0.04 (-0.05)	9.9 ± 0.64 (-0.52)	98.5 ± 2.51 (-1.53)	0.050 ± 0.01 (0.50)	0.95 ± 0.05 (-5.08)	9.68 ± 0.15 (-3.17)
Long-term stability ^b (n=4)	0.52 ± 0.02 (4.5)	NA	100.3 ± 5.09 (0.3)	0.058 ± 0.003 (15.5)	NA	10.0 ± 0.34 (0.3)

^aShort-term stability, 24h in room temperature (20°C); ^bLong-term stability, 12 months in -75°C; %RE: Relative error from nominal concentration.

Table 2: Stability analysis of the analytical method for the measurement of morphine and morphine-6-glucuronide concentrations in human plasma.

concentration from patients during intensive care treatment and receiving several other drugs concomitantly.

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