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Development and validation of a sensitive method for hydromorphone in human plasma by normal phase liquid chromatography–tandem mass spectrometry

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Abstract

Liquid chromatography coupled with tandem mass spectrometry (LC–MS–MS) was developed for the quantitation of hydromorphone (HYD), an opiate analgesic, in human plasma. A simple liquid–liquid extraction was used to extract the analyte and its deuterated internal standard (d_3-HYD). Chromatographic separation of hydromorphone from its metabolite hydromorphone-3-glucuronide (H3G) was necessary because of the significant H3G fragmentation to HYD before Q1 of the mass spectrometer, which could result in false detection as HYD in the multiple reaction mode (MRM). This separation was achieved using a 50×2 mm, I.D. silica column (5 μm) and a mobile phase of acetonitrile–water–formic acid (80:20:1, v/v/v). The method was validated in the concentration range 0.05–10 ng ml^{-1} in plasma and met the acceptance criteria of industry guidelines for accuracy, precision, and stability.

Keywords: Hydromorphone; Opiate; Normal phase liquid chromatography; Tandem mass spectrometry; In-source fragmentation

1. Introduction

Hydromorphone (HYD) is a synthetic opiate analgesic and is often used for pain management for post-operation and cancer patient. HYD is metabolized via conjugation to hydromorphone-3-glucuronide (H3G) and excreted in the urine. Fig. 1 shows the chemical structures of HYD and H3G. Approximately 35% were reported to be H3G in the urine of patients after per oral administration, and ~2% in the form of 6α- and 6β-hydroxyl conjugates [1,2]. Plasma H3G concentrations after dosing can be much higher than HYD at certain time point [3]. Analysis of HYD and other opiate analgesics have been performed by immunoassays or by HPLC methods [3–6]. Immunoassays lack selectivity while HPLC methods may not have adequate sensitivity. Liquid chromatography coupled with tandem mass

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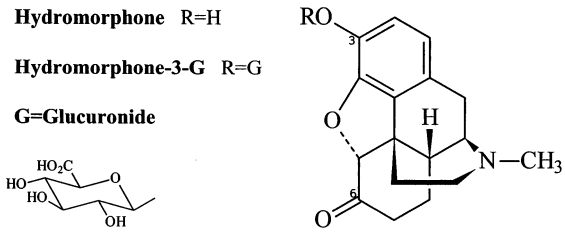

Fig. 1. Chemical structures of hydromorphone and hydromorphone-3-glucuronide.

spectrometry (LC–MS–MS) has been proved to be the method of choice because of its sensitivity, selectivity and analysis speed. LC–MS–MS methods for morphine (MOR) and its conjugated metabolites, morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G) have been published [7–15]. So far, no LC–MS–MS methods were reported in the literature for the quantitation of HYD in biological fluids.

In order to analyze large number of samples generated from clinical studies, a fast and specific LC–MS–MS method was pursued. The separation between HYD and H3G is paramount important since the H3G concentrations after dosing can be much higher than HYD at certain time points and H3G could be fragmented to HYD prior to Q1 and falsely detected as HYD in the MRM mode [16]. We developed a method with normal phase chromatography using silica column as stationary phase and a mobile phase consisting of acetonitrile, water and formic acid. This method provided an excellent separation between HYD and H3G in 2.5 min. This method

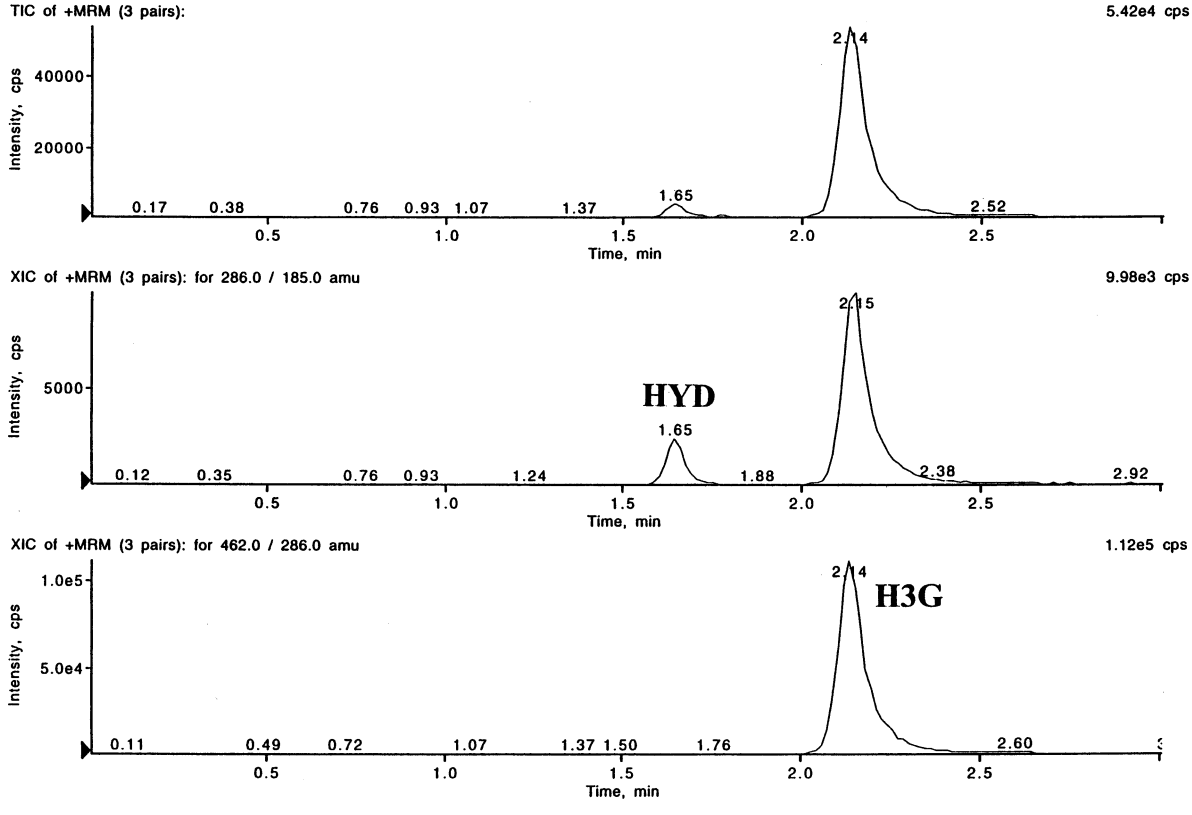

Fig. 2. Presence of hydromorphone m/z ions at the retention time of hydromorphone-3-glucuronide. A solution of H3G and HYD mixture was injected onto the LC–MS–MS. Column: Inertsil Silica column 50 × 2 mm, I.D.; Mobile phase: acetonitrile–water–formic acid (80:20:1, v/v/v); Flow rate: 0.2 ml min^{-1}; Injection volume: 20 μl. Top panel: total ion current; middle panel: HYD channel; bottom panel: H3G channel. Retention time: HYD, 1.65 min; H3G, 2.14 min.

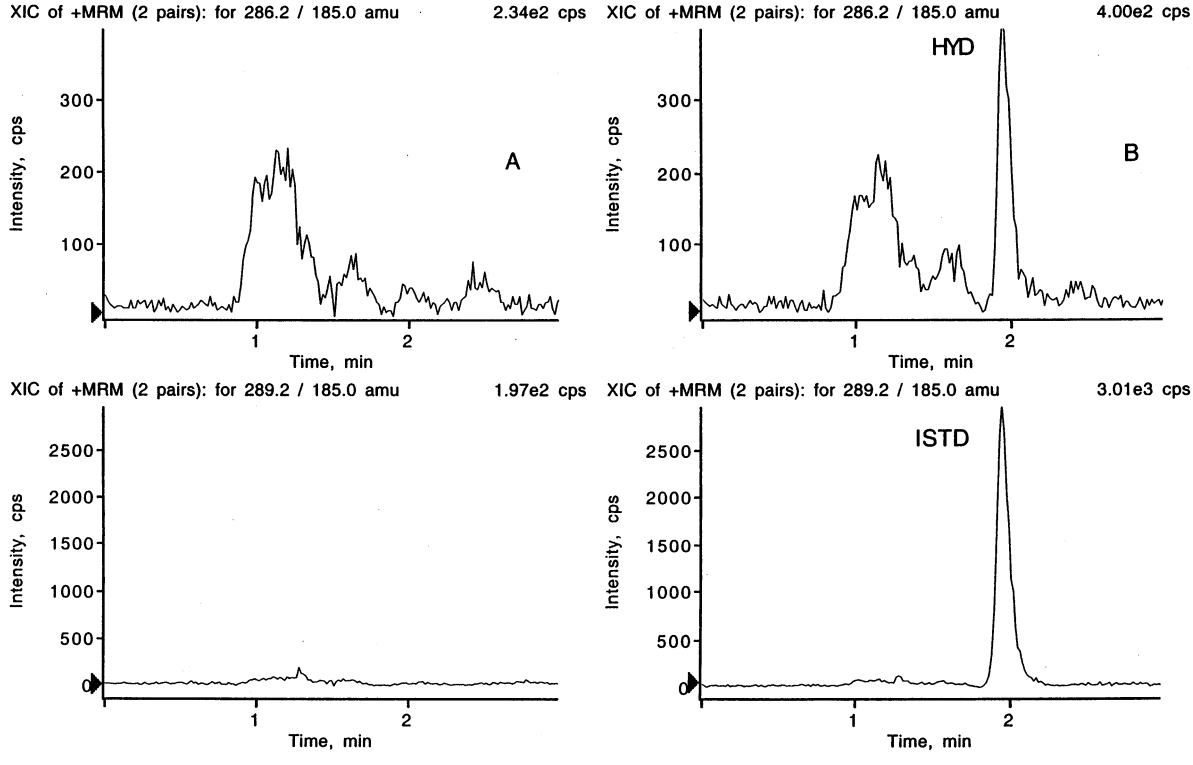

Fig. 3. Chromatograms of HYD in blank control plasma (panel A) and at 0.05 ng ml^{-1} (panel B). See Fig. 2 for the chromatographic conditions and experimental section for the sample extraction.

was validated according to the industry guidelines.

2. Experimental

2.1. Chemicals

Hydromorphone hydrochloride was purchased from United States Pharmacopeia (Rockville, MD). Deuterated internal standard (d_3-HYD) was from Radian (Austin, TX). H3G was gift from Purdue Federick (Norwalk, NJ). All organic solvents were of HPLC grade and were from Fisher (St. Louis, MO). HPLC grade water and formic acid were also from Fisher. All inorganic salts were of analytical grade and were from Mallinckrodt (Irvine, CA). Human EDTA plasma were drawn in-house from healthy volunteers.

2.2. Chromatographic conditions

The LC system consisted of a LCD 3500 pump (Riviera Beach, FL) and a Waters 717 autosampler (Milford, MA). Normal-phase LC was performed on an Inertsil silica column (50×2 mm I.D., 5 μm) from Keystone (Bellefonte, PA). The mobile phase was acetonitrile–water–formic acid (80:20:1, v/v/v). The column was maintained at ambient temperature and a constant flow-rate of 0.2 ml min^{-1} was employed. The injection volume was 20 μl. Equilibration with the mobile phase for ~ 0.5 h was performed for a brand new column. The column then required only ~ 5–10 min of equilibration time before each use. The column never needed washing between curve runs. One column can be used for at least 500 injections.

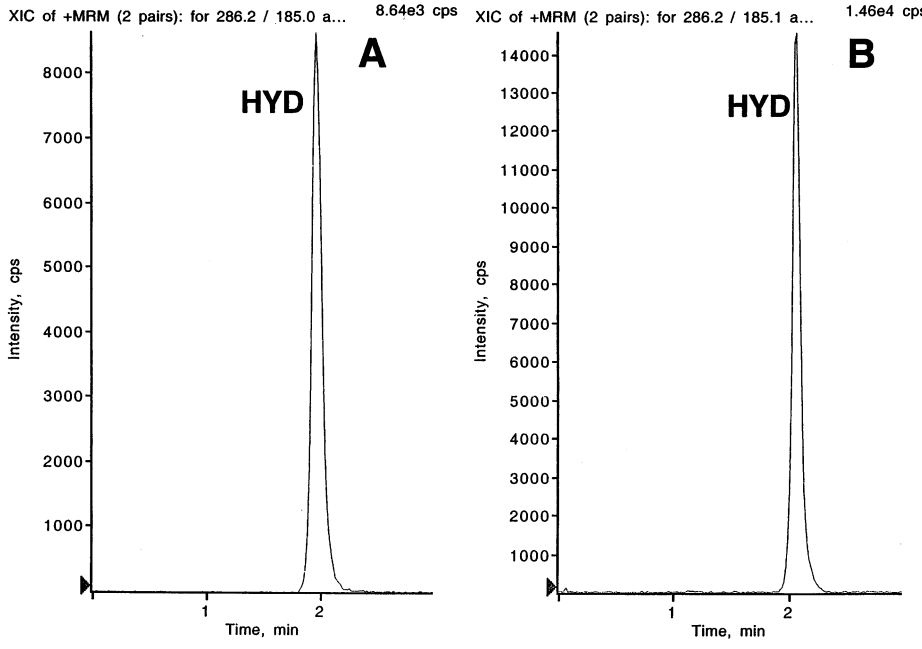

Fig. 4. Chromatograms of HYD at first injection (panel A).vs. injection #86 (panel B). See Fig. 2 for the chromatographic conditions and experimental section for the sample extraction.

Table 1
Accuracy and precision of hydromorphone in plasma standards[a,b]

	Hydromorphone (ng ml^{-1})								
Theoretical conc.	0.0500	0.100	0.200	0.500	1.00	2.00	5.00	8.00	10.0
Calculated mean conc.	0.0490	0.101	0.203	0.518	0.987	1.98	5.06	8.01	9.65
RSD%	4.1	7.9	3.5	2.7	3.9	2.2	2.4	2.2	3.5
RE%	−2.0	+1.0	+1.5	+3.6	−1.3	−1.0	+1.1	+0.1	−3.5

[a] Interday statistics from six standard curve runs
[b] RSD, relative standard deviation; RE, relative error.

Table 2
Accuracy and precision of hydromorphone in plasma quality control samples[a]

	Hydromorphone (ng ml^{-1})					
	Interday ($n=36$)			Intraday ($n=6$)		
Theoretical conc.	0.150	1.50	7.50	0.150	1.50	7.50
Calculated mean conc.	0.148	1.51	7.40	0.140	1.53	7.40
RSD%	5.4	3.3	3.9	5.0	2.2	2.4
RE%	−1.3	+0.3	−1.4	−6.7	+1.9	−1.3

[a] RSD, relative standard deviation; RE, relative error.

Table 3
Stability of hydromorphone in plasma and sample extracts

Sample extract	Time period	Percentage of control
Reinjection/Refrigeration	60 h	97–105
Plasma sample		
Benchtop at room temperature	24 h	101–104
Freeze/thaw	3 cycles	97–101
Sample storage (−20°C)	10 days	96–101

2.3. Mass spectrometric conditions

A Perkin Elmer Sciex API 3 plus triple quadrupole mass spectrometer equipped with an API source (Thornhill, Ontario, Canada). The API source was fitted with an ESI inlet for ionizing the analytes in the HPLC eluent. Turbo ionization was achieved by applying a spray voltage of + 3.5 kV. The source temperature was 400°C. High purity nitrogen served as drying gas at a flow-rate of 4.0 l min^{-1}. MRM was used for the detection of HYD and d_3-HYD with a dwell time of 350 ms for both compounds. The first quadrupole (Q1) was set up to transmit the molecular ions MH^+ at m/z 286 (HYD) and 289 (d_3-HYD). These molecular ions were fragmented by collision activated dissociation with argon at − 25 eV in the second quadrupole (Q2). The product ions were monitored in the third quadrupole (Q3) at m/z 185 for both HYD and d_3-HYD.

2.4. Preparation of solutions

Primary standard and quality control stocks of approximately 400 μg ml^{-1} were prepared from separate weighing. Working standards were prepared in water by diluting the primary stock solution. These working standards were stable for at least 30 days when stored at 2–8°C in a polypropylene tube. Daily calibration standards were prepared by spiking 0.1 ml of 10-X working standards into 1.0 ml of blank control EDTA plasma, resulting final concentrations of 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 8, and 10 ng ml^{-1} in plasma. Quality control samples (QCs) at concentrations of 0.15, 1.5 and 7.5 ng ml^{-1}, were prepared by spiking a small volume (< 1% of total volume) of stock solutions into blank control EDTA plasma. QCs were aliquoted in 2.25 ml volumes into polypropylene tubes and were stored at − 20°C until assayed. Working internal standard (IS) solution of d_3-HYD was prepared in water at 50 ng ml^{-1}.

2.5. Sample processing

To a 16 × 100 mm glass screw-cap tube, 0.1 ml of appropriate working standard or 0.1 ml of water for QCs was added. A total of 1 ml of QCs or analytical sample, or blank control EDTA plasma for the standards, was then added. After mixing by vortex, 0.1 ml of working IS was added to all tubes except the blank control sample. Subsequently, 1 ml of 100 mM ammonium phosphate buffer pH 8.6 was added and the tubes were vortexed for 1 min. The analytes were extracted into 8 ml of methyl-tertiary butyl ether by shaking the tubes on a horizontal shaker vigorously for 10 s. After centrifuging for 5 min, the bottom (aqueous) layer was frozen in a dry-ice/acetone bath and the upper organic layer decant into a silanized, 13 × 100 mm glass culture tubes. The organic extract was evaporated to dryness under nitrogen at 50°C, and the residue reconstituted in 75 μl of acetonitrile by vortexing for 3 min. The extract was then transferred to injection vials containing glass inserts with elevation legs.

2.6. Conduct of validation

A standard curve was defined by one set of calibration standards, placed near the beginning of each run. Six standard curves assayed over a period of 9 days determined the inter-day and intra-day reproducibility. QCs were also run with the standard curves in achieving inter-day, and intra-day data. Stability of the analyte in plasma and through the analytical process (storage, freeze/thaw, bench-top and re-injection) was established with QCs.

2.7. Analytical data treatment

Chromatograms were measured using a MacQuan™ data system, and subsequently transferred into the VAX/VMS® Oracle database. A weighted [$(1/x^2)$ where x is the concentration of HYD] linear regression was used to determine slopes, intercepts, and correlation coefficients. The resulting parameters were used to calculate concentrations:

$$\text{Concentration} = [\text{Ratio} - (y - \text{intercept})]/\text{Slope}$$

where 'ratio' is the ratio of the HYD peak area to the IS peak area.

3. Results and discussion

3.1. Chromatography and mass spectrometry

When a sample of only H3G was injected onto the LC–MS–MS system the HYD channel showed a peak at the retention time of H3G. This peak was the result of H3G conversion to HYD by fragmentation before Q1; the deconjugated fragment was then detected in the MRM channel of HYD. The magnitude of the fragmentation was significant as shown in Fig. 2, estimated to be 10%, by comparing peak areas of H3G in both the H3G and HYD channels. Depending on the sample clean-up, variable amounts of H3G may remain in the sample extract. Because the H3G concentration could be much higher than HYD in clinical samples, the residual amount of H3G in the extract may be substantial. In order to assure that H3G will not interfere with the quantitation of free HYD, chromatographic resolution of H3G from HYD was necessary. In addition to HYD and H3G, fragmentation of the glucuronide conjugates of morphine before Q1 has also been reported [17].

One of the primary goals of this study was chromatographic separation of HYD from H3G because of the in-source fragmentation of H3G to HYD. With reversed-phase HPLC system, we experienced three challenges to achieve this goal. Firstly, the separation was not readily obtainable within a short run time. Secondly, the low organic content ($< 20\%$) in the reversed-phase HPLC system was necessary to retain the compounds on the column. Mobile phases of low amount of organic modifier will adversely affect the sensitivity due to the poor spray condition [16], while a retention of the analytes on the column is necessary to minimize the matrix effects [18–22]. Thirdly, in order to produce preformed ion for HYD which contains an amino functional group, an acidic mobile phase was preferred. Preformed ions were recommended for good sensitivity [23]. However, the protonated HYD, being a more polar species than HYD, will even be poorly retained on a reversed-phase HPLC column. This means that a lower organic content in the mobile phase will be needed to retain the compound, resulting in poor MS sensitivity. Therefore, we pursued the use of a normal phase silica column with a mobile phase of high organic solvent and an acid modifier to solve the problems presented by above challenges. As shown in Fig. 2, excellent separation was achieved for H3G and HYD within 2.5 min under normal phase conditions. Retention time was 2.15 min for H3G and 1.65 min for HYD. The mobile phase for the normal phase consisted of mostly organic solvent, acetonitrile and formic acid. This high organic solvent content provided a fine spray condition at the interface, resulting in higher sensitivity and more stable MS signal throughout an analytical run. Fig. 3 shows sensitivity and selectivity against endogenous matrix background. The signal to noise ratio (S/N) was at least 5:1. In addition to MS signal stability, the silica column had demonstrated excellent stability under this kind of normal phase condition as illustrated on Fig. 4. The retention time and peak symmetry of the initial injection versus injection # 86 were the same. No retention time shifting and peak shape deterioration was observed. The same analytical column could be used for at least 500 injections. The column back pressure was very low with the normal phase chromatography. On the 50×2 mm, I.D. column, the back pressure was only about 150 psi at 0.2 ml min^{-1}.

3.2. Accuracy and precision

Accuracy and precision was established from six analytical runs for standards (Table 1) and QCs (Table 2). The standard curve for plasma samples was linear over the concentration range 0.05–10 ng ml^{-1} with a correlation coefficients of $r^2 > 0.998$. The RSD from six validation analytical runs over 10 days was $\leq 7.9\%$ for spiked plasma standards and $\leq 5.4\%$ for QCs. The within-day variation of QCs were $\leq 5.0\%$. The relative errors (RE) for interday and intraday QCs were ≤ 1.4 and 6.7% respectively. The slopes of the standard curves through the validation were very consistent (2.35–2.53).

3.3. Sensitivity and selectivity

As shown in Fig. 3, the LOQ (50 pg ml^{-1}) showed signal to noise of at least 5:1. With five replicates of the LOQ sample of the same plasma lot, %RSD was 4.1, and %RE was -2.0. The recoveries were relatively low, $\sim 25\%$ for HYD. However, due to the use of deuterated d_3-HYD, the d_3-HYD tracked HYD very consistently as indicated by tight %RSD for ratios of the standards and QCs. Besides H3G, the method was also tested against another minor metabolite of HYD, namely dihydromorphine. No interference was observed. Six out of six lots of control plasma were tested and found to be free from interference for the compound.

3.4. Stability

Stability tests of the analyte in plasma and in sample extracts were established. The results are shown in Table 3. The analytes were stable during storage, sample extraction process and chromatography.

4. Conclusion

A normal phase LC–MS–MS method was developed and validated for the quantitation of hydromorphone in human EDTA plasma. A simple liquid–liquid extraction was used. Hydromorphone was well separated from its conjugated metabolite hydromorphone-3-glucuronide on an Inertsil silica column (50 × 2 mm, I.D.) with a mobile phase of acetonitrile–water–formic acid (80:20:1, v/v/v) within 2.5 min. This separation was crucial since fragmentation of hydromorphone-3-glucuronide to hydromorphone in the source could result in an over-estimation of hydromorphone. The advantages of using normal phase LC–MS–MS with polar mobile phase over reversed-phase LC–MS–MS have been demonstrated. The method was validated according to the industry guidelines for selectivity, sensitivity, linearity, accuracy, precision and stability [24].

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