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Short communication

Development and validation of a high performance liquid chromatography–tandem mass spectrometry for the determination of etodolac in human plasma

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Abstract

A simple and specific method using a one-step liquid–liquid extraction (LLE) with butyl acetate followed by high performance liquid chromatography (HPLC) coupled with positive ion electrospray ionization tandem mass spectrometry (ESI-MS/MS) detection was developed for the determination of etodolac in human plasma, using indomethacin as an internal standard (IS). Chromatographic separation was performed isocratically using a Capcellpak MGII C_{18} column with 65% acetonitrile and 35% water containing 10 mM ammonium formate (adjusted to pH 3.5 with formic acid). Acquisition was performed in multiple reaction monitoring (MRM) mode by monitoring the transitions: *m/z* 287.99 > 172.23 for etodolac and *m/z* 357.92 > 139.01 for IS. The method was validated to determine its selectivity, linearity, sensitivity, precision, accuracy, recovery and stability. The limit of quantitation (LLOQ) was 0.1 μg/mL with a relative standard deviation of less than 15%. The devised method provides an accurate, precise and sensitive tool for determining etodolac levels in plasma.

Keywords: Etodolac; Indomethacin; LC–MS/MS; Human plasma; Validation

1. Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are used in humans and domestic animals due to their anti-inflammatory, analgesic and anti-pyretic effects [1]. Etodolac (1,8-diethyl-1,3,4,9-tetrahydropyrano[3,4-*b*]-indole-1-acetic acid) is an effective and well-tolerated NSAID, and is indicated for the treatment of analgesia and for the signs and symptoms of rheumatoid arthritis and osteoarthritis [2]. In rats, etodolac exhibits a greater therapeutic index between anti-inflammatory activity and gastric irritation than do many other NSAIDs [3]. In patients with rheumatoid arthritis, etodolac at doses of 100–300 mg given twice daily is more efficacious than aspirin or a placebo [4]. In healthy adults, orally administered etodolac is rapidly absorbed, with a time to peak plasma drug concentration (t_{max}) of approximately 1 h for an immediate release formulation. Like other NSAIDs, etodolac is highly (99.3%) bound to plasma protein, and in plasma, etodolac exhibits a mean apparent volume of distribution (Vd/F) of 0.39 L/kg, an oral clearance (CL/F) of 0.049 L/(h kg) and an elimination half-life ($t_{1/2}$) of approximately 7 h. Etodolac is extensively metabolized in the liver to inactive oxidative metabolites, which are primarily eliminated via the renal route [5].

Earlier publications have described methods of analyzing etodolac in biological samples. The techniques adopted include; spectrometry and spectrofluorimetry [6], gas chromatography (GC) [7], high performance liquid chromatography (HPLC) [8,9] and gas chromatography–mass spectrometry (GC–MS) [10]. However, these methods suffer from a number of disadvantages, a large volume of plasma (≥0.02 mL) [7–10], lengthy run times (>3 min) [7–10], the lack of an internal standard (IS) [8,9] and lower sensitivity, which can more accurate analysis (0.1 μg/mL) [8–10]. Although capillary

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electrochromatography–electrospray ionization mass spectrometry (CEC–ES-MS) and LC–ESI-MS methods have been reported [11,12], no method has been described in the literature for the detection of etodolac by liquid chromatography/tandem mass spectrometry (LC/MS/MS) to our knowledge.

The aim of this study was to develop a more sensitive liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) system with a 100 ng/mL lower limit of quantification (LLOQ) and a 3 min run time, for determining etodolac levels in human plasma to support pharmacokinetic studies.

2. Experimental

2.1. Materials and reagents

Etodolac, indomethacin and dimethyl sulfoxide were obtained from Sigma–Aldrich (St. Louis, MO, USA) and butyl acetate and acetonitrile (HPLC grade) from J.T. Baker (Philipsburg, NJ, USA). Water was purified using a Milli-Q purification system (Millipore Corp., Bedford, MA, USA). All other chemicals and solvent were of the highest analytical grades available.

2.2. Calibration standard and quality control samples

A stock solution of etodolac was prepared in 50% acetonitrile (50% acetonirile containing purified water, v/v) at 1000 μg/mL. This stock solution was appropriately diluted with 50% acetonitrile to obtain working standards for calibration purposes. Calibration curves of etodolac were prepared by spiking blank plasma at 0.1, 0.25, 0.5, 1, 2.5, 5, 10 and 25 μg/mL. Quality control (QC) samples (0.6, 3 and 15 μg/mL) were also prepared by diluting stock solution. In order to prepare stock solutions (1000 μg/mL) of IS, 10 mg of indomethacin was dissolved in 10 mL of dimethyl sulfoxide, and further diluted with 50% acetonitrile to a final concentration of 5 μg/mL. All solutions were stored at −80 °C.

2.3. Instrumentation and chromatographic conditions

A Waters 2795 HPLC system and a Waters Micromass Quattro Premier triple quadrupole mass spectrometer equipped with a turbo electrospray interface in positive ionization mode (Waters Ltd., Watford, UK) were used for the LC–MS/MS analysis. Two channels of positive ion MRM mode were used to detect etodolac and IS. The most abundant product ions of the compounds were at *m/z* 172.23 from the parent *m/z* 287.99 ion of etodolac and at *m/z* 139.01 from the *m/z* 357.92 ion of IS. Data acquisition was performed using Micromass Masslynx 4.0 and data processing was conducted using a Quanlynx data analysis program.

The analytical column used was a Capcellpak MGII C_{18} (50 mm × 2.0 mm i.d. 3 μm, 12 nm; Shiseido, Kyoto, Japan), and the mobile phase consisted of 65% acetonitrile and 35% Milli-Q water containing 10 mM ammonium formate (adjusted to pH 3.5 with formic acid), and was filtered through a 0.2 μm filter and degassed before use. A flow rate of 0.2 mL/min was used for sample analysis. The temperatures of the autosampler and column oven were 4 and 40 °C, respectively.

2.4. Chromatographic conditions robustness

To evaluate the robustness of the analytical method, the effects of the most important chromatographic parameters, e.g., flow rate, column temperature and mobile phase pH, were deliberately changed. Each parameter was studied at a high level (flow 0.3 mL/min; column temperature 45 °C; mobile phase pH 2.5), an intermediate level (flow 0.2 mL/min; column temperature 40 °C; mobile phase pH 3.5) and low level (flow 0.1 mL/min; column temperature 35 °C; mobile phase pH 4.5) with respect to the value specified in the analytical method. C_{18} columns were examined from two different suppliers (Hydrosphere-C_{18} [YMC; Kyoto, Japan] and Capcellpak MGII [Shiseido; Kyoto, Japan]).

2.5. Sample preparation

Plasma samples were stored at −80 °C and allowed to thaw at room temperature before processing. Briefly, a plasma sample (20 μL) was placed in a 1.7 mL Eppendorf tube, and after adding 20 μL of a 5 μg/mL solution of IS was vortexed briefly. 1.5 mL of butyl acetate was then added to each sample, shaken for 10 min, and centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant organic layer (1 mL) was evaporated to dryness in Savant SC-110A Speed Vac system (Savant instruments, Holbrook, NY, USA). The residue obtained was dissolved in 1.5 mL of 50% acetonitrile (acetonitrile/water; 1:1 v/v) and vortexed for 10 min. After centrifugation for 5 min at 14,000 rpm, the sample was transferred to an autosampler vial and 10 μL was injected into the LC–MS/MS system.

2.6. Method validation

The method was validated in terms of selectivity, linearity, sensitivity, precision, accuracy, recovery and stability according to the guidelines issued by the food and drug administration (FDA) for the validation of bioanalytical methods [13].

2.6.1. Selectivity

Blank plasma samples from eight different volunteers were tested for the presence of endogenous compounds that might interfere with analyte, using the proposed extraction procedure and chromatographic/spectroscopic conditions, and results were then compared with those obtained with a solution of the analyte at a concentration near the LLOQ.

2.6.2. Calibration curve, linearity and sensitivity

A calibration curve was constructed using a double-blank sample (a plasma sample without etodolac and IS) and seven calibration samples covering the whole range (0.1–25 μg/mL) by the peak area ratio of etodolac against IS. Concentrations of etodolac were calculated from these area ratios using the calibration curve. The linearity of the calibration curve was also calculated, and a correlation coefficient (r^2) of 0.99 or better was

deemed satisfactory. LLOQ was defined as the lowest concentration with a coefficient of variance (CV) and relative standard deviation (R.S.D.) of 20%.

2.6.3. Precision and accuracy

Intra- and inter-day assay precisions were determined as coefficient of variance (CV), and intra- and inter-day assay accuracies were expressed as percentages of the theoretical concentration, as accuracy (%) = (found concentration/theoretical concentration) × 100. Intra-day assays were performed using five replicates during 1 day and inter-day assays were performed on four separate days. The acceptance criterion recommended by FDA for each back-calculated standard concentration was a 15% deviation from the normal value except at the LLOQ, which was set at 20% [13].

2.6.4. Recovery and matrix effect

For evaluation of absolute recovery (ARE) and absolute matrix effect (AME; ion suppression or enhancement), three sets of QC samples (0.6, 3.0 and 15.0 μg/mL, $n = 3$) were prepared by the preparative procedure described above with IS. Pure solution set was prepared to measure the MS/MS response of neat standard solutions in mobile phase without any matrix. Pre-extraction set was prepared by the proposed preparative procedure. Post-extraction set was prepared by dissolving analytes in the extracted blank sample (the solution of blank plasma reconstructed with pure solution after extraction). The corresponding peak areas of analyte in pure solution set (A), were then compared to those of the pre-extraction set (B) or post-extraction set (C) at equivalent concentrations. The ratio of B/A is defined as the ARE, and the ratio of C/A is defined as the AME. The value was expressed as percentage. The AME value of >100% indicates ionization enhancement, and the AME value of <100% indicates ionization suppression. To evaluate relative matrix effect (RME), blank plasma samples (eight lots) from eight different volunteers were used to prepare QC samples (0.6, 3.0 and 15.0 μg/mL). For each lot, triplicate samples were analyzed at each concentration. The CV of the peak area for etodolac was calculated to determined inter-lot matrix variability.

2.6.5. Stability

The stability of etodolac was assessed by analyzing QC samples exposed to different temperatures for different times. QC samples were prepared in sufficient volume to allow multiple replicates ($n = 5$) at each test condition. Results were compared with those of freshly prepared QC samples, and percentage concentration deviations were calculated. Stability was calculated as the difference from the freshly prepared samples. The protocol for the stability study included short- and long-term stability. Short-term stability included: (a) freeze–thaw stability, which was determined after three freeze–thaw cycles on consecutive days; (b) exposure of samples to room temperature for 6 h; (c) exposure of samples to room temperature for 1 and 2 days; (d) exposure to 4 °C (refrigerator) for 1 day; (e) exposure to −80 °C (deep freezer) for 1 day; (f) dilution of samples two and four times using blank plasma; (g) the exposure to mobile phase at room temperature for 12 h. Long-term stability included: (a) analysis of samples, which were frozen and thawed after 30 days at −80 °C; (b) stability of etodolac in mobile phase after refrigeration (4 °C) for 20 days.

3. Results and discussion

3.1. Optimization of MS detection and chromatographic conditions

Mass spectrometric parameters were optimized to achieve the maximum abundances of product and fragment ions. The main instrument parameters of the mass spectrometer are summarized in Table 1. Full scan mass spectra and product ion scan spectra of etodolac and IS were obtained by direct infusion into the mass spectrometer of 1 μg/mL diluted in mobile phase at a flow rate of 0.2 mL/min. Etodolac and IS mass spectra exhibit protonated species $[M+H]^+$ at *m/z* 287.99 and *m/z* 357.92, respectively, which were chosen as precursor ions. The two compounds fragmented to produce intense product ion signals at *m/z* 172.23 and *m/z* 139.01, respectively. Ionization and fragmentation were found to be highly efficient, and as a result, a substantial detection response was obtained at the lower limit of quantitation (0.1 μg/mL). Full scan and product ion mass spectra of etodolac and IS are displayed in Fig. 1(a) and (b), respectively. Robustness studies showed that small variations in flow rate, column temperature and analytical C_{18} columns did not affect the resolution of etodolac during analysis. However, mobile phase pH influenced the resolution of etodolac and the most sensitive was at pH 3.5 (data not shown).

Table 1
Main operating parameter of the LC–MS/MS

Parameter	Value
Source temperature (°C)	100
Desolvation temperature (°C)	350
Desolvation gas flow (h^{-1})	500
Cone gas flow (h^{-1})	0
Collision cell gas pressure (mbar)	3.03×10^{-3}
Capillary voltage (kV)	3.14
Extractor (V)	2
RF lens (V)	0.4
Low mass 1 resolution	14
High mass 1 resolution	14
Ion energy 1 (V)	2
Low mass resolution	14
High mass resolution	14
Ion energy 2 (V)	1
Entrance (V)	24
Exit (V)	2
Multiplier	650
Dwell time (s)	0

	Etodolac	IS
Molecule ion (*m/z*)	287.99	357.92
Product ion (*m/z*)	172.23	139.01
Cone energy (V)	19	28
Collision energy (V)	24	15

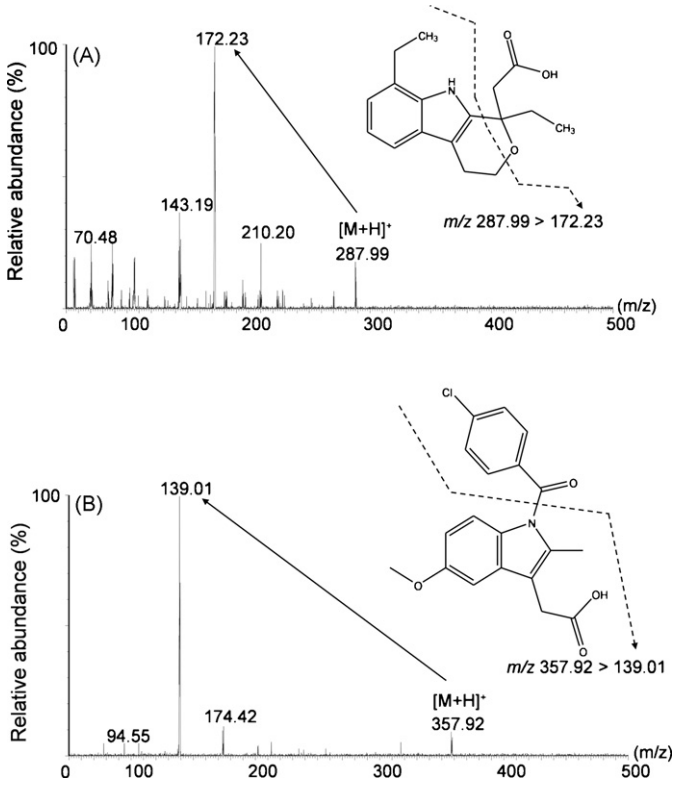

Fig. 1. Chemical structures and product ion mass spectra of the $[M+H]^+$ ions of (A) etodolac and (B) indomethacin (IS).

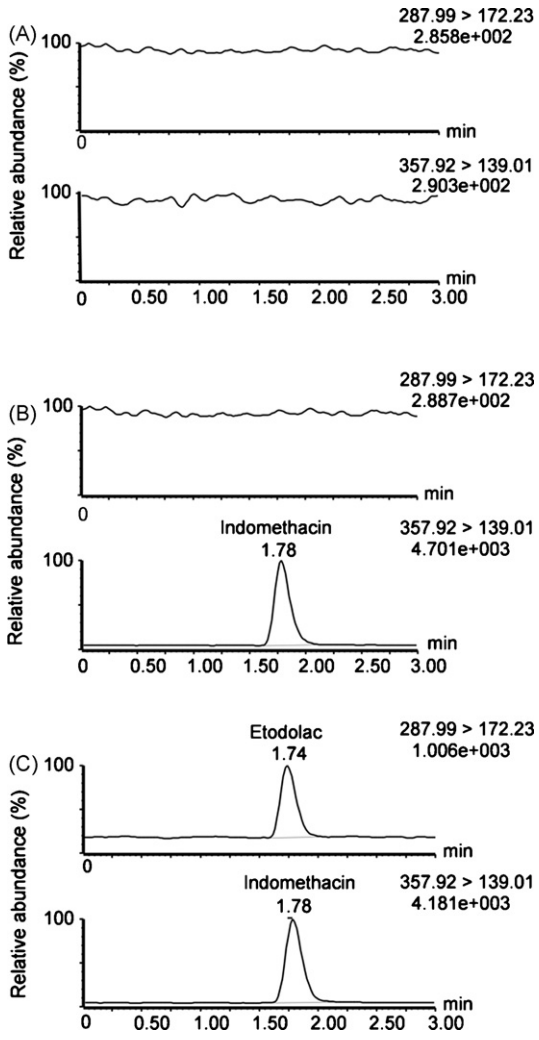

Fig. 2. MRM chromatograms resulting from analyses of (A) blank (drug and IS free), (B) zero blank (drug-free spiked with the IS) human plasma and (C) 0.1 μg/mL (LLOQ) of etodolac spiked with IS.

3.2. Method validation

3.2.1. Selectivity

Method selectivity was investigated by preparing and analyzing human plasma blanks from six different batches of pooled human plasma, blank human plasma extract (Fig. 2(a)), extract spiked only with IS (Fig. 2(b)), and extract spiked with etodolac and IS (Fig. 2(c)). As shown in Fig. 2(a), no endogenous peaks were observed at the retention times of etodolac or IS. Fig. 2(c) shows MRM chromatograms at the LLOQ (0.1 μg/mL) of the calibration curve. As shown, the chromatogram retention times for etodolac and IS were 1.74 and 1.78 min, respectively. The total LC/MS/MS analysis time was 3 min per sample.

3.2.2. Linearity and sensitivity

The eight-point calibration curve exhibited good linearity in the concentration range of 0.1–25.0 μg/mL. The linear regression equation of the analyte calibration curve was $y = 1.1191(\pm 0.0312) + 0.0949(\pm 0.0183)$ ($r^2 = 0.9975 \pm 0.0018$), where y is the peak area ratio of the etodolac to IS and x is the concentration of the etodolac. The LLOQ for etodolac was proved to be 0.1 μg/mL. Calibration curves were obtained without a weighting factor.

3.2.3. Precision and accuracy

Precision was calculated as CV and accuracy was always below 15% in the concentration range of 0.1–25 μg/mL with butyl acetate extraction procedure. The intra-day CV of the method for determining etodolac ranged from 1.3% to 6.7%, and its accuracy ranged from 89.3% to 106.3%. The inter-day CV ranged from 1.9% to 6.3%, and accuracy from 90.3% to 105.3% (Table 2). These results satisfied FDA criteria and demonstrated the reproducibility of the devised method.

Table 2
Intra- and inter-day assay precision and accuracy for LC–MS/MS assay of etodolac in human plasma ($n = 5$ per test)

Theoretical concentration (μg/mL)	Accuracy (%)		Precision (CV%)	
	Intra-day	Inter-day	Intra-day	Inter-day
0.1	95.84	95.67	6.69	4.37
0.5	106.13	105.04	1.29	2.45
1	104.72	104.13	1.85	6.31
5	106.25	105.27	1.87	4.83
10	91.25	94.29	1.73	2.29
25	89.32	90.31	2.45	1.88

Table 3
Absolute recovery (ARE) and absolute matrix effect (AME) and relative matrix effect (RME) of etodolac

Theoretical concentration (μg/mL)	ARE mean ± S.D. (%)	AME mean ± S.D. (%)	RME	
			Mean ± S.D. (%)	CV%
0.6	91.12 ± 3.09	92.19 ± 0.06	0.643 ± 0.601	9.30
3	92.58 ± 0.54	105.47 ± 0.17	3.160 ± 2.939	9.89
15	98.81 ± 0.07	95.53 ± 1.67	18.697 ± 16.298	9.89

3.2.4. Recovery and matrix effect

As shown in Table 3, the AREs following extraction at three different concentrations of etodolac were about 90%. The AMEs for etodolac was more than 90% and the RME for etodolac was less than 10%. These results indicate that no co-eluting endogenous substances significantly influenced the ion suppression in this analytical method. Accordingly, it is considered that the proposed analytical method is reliable and is subject to minimal matrix effect.

3.2.5. Stability

Studies were performed to determine drug stability in plasma and in the mobile phase used for preparing standard solutions, and no degradation was evident (data not shown). These results indicate that etodolac was stable under bench (room temperature), auto sampler (4 °C) and freeze–thaw conditions, and importantly no stability-related problems were encountered during routine sample analysis.

4. Conclusions

The LC–MS/MS method developed for the determination of etodolac in human plasma, based on a simple liquid–liquid extraction and isocratic chromatography, proved to be rapid and sensitive. Moreover, the devised method fully meets FDA guidelines [13], and has high sensitivity, reproducibility and specificity.

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