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A liquid chromatography method with single quadrupole mass spectrometry for quantitative determination of indomethacin in maternal plasma and urine of pregnant patients

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Abstract

A liquid chromatography with single quadrupole mass spectrometry method was developed for the quantitative determination of indomethacin in the maternal plasma and urine of pregnant patients under treatment. A deuterium-labeled isotope of indomethacin (d_4 -indomethacin) was used as an internal standard. The maternal plasma and urine samples were acidified with 1.0 M HCl then extracted with chloroform to achieve the extraction recovery range of 94% to 104% with variation less than 11%. Chromatographic separation was achieved by a Waters Symmetry C₁₈ column with isocratic elution of 0.05% (v/v) formic acid aqueous solution and acetonitrile (47:53, v/v). An in-source fragmentation was applied on the single quadrupole mass spectrometer equipped with an electrospray ionization source at positive mode. The LC-ESI-MS quantification was performed in the selected ion monitoring mode targeting ions at m/z 139 for indomethacin and m/z 143 for its internal standard. The calibration curves were linear in the concentration ranges between 14.8 and 2.97×10^3 ng/mL for plasma samples and between 10.5 and 4.21×10^3 ng/mL for urine samples. The relative standard deviation of this method was less than 8% for intra- and inter-day assays, and the accuracy ranged between 90% and 108%.

Keywords

Indomethacin; LC-MS; plasma; urine; method validation

1 Introduction

Preterm birth is a major cause of neonatal mortality and morbidity worldwide. In the United States, the rate of preterm delivery is approximately 11% of live births [1, 2]. Indomethacin, a prostaglandin synthetase inhibitor, is considered as a first-line tocolytic agent, beside magnesium sulfate, for the management of preterm labor [3]. However, indomethacin is classified by the FDA of the United States as pregnancy category C because of inadequate data on its safety and efficacy in this population. Therefore, one of the aims of the recent

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investigation by the NICHD Obstetric-Pharmacology Research Network (OPRU) is to determine the pharmacokinetics (PK) of indomethacin in pregnant patients.

A number of analytical methods for the quantitative determination of indomethacin in human biological samples have been reported. These methods have included liquid chromatography with UV (LC-UV) [4–12] and fluorometric detection [13] as well as liquid chromatography-mass spectrometry (LC-MS/MS) [14]. The reported HPLC-UV methods either required larger volumes (> 0.4 mL) of plasma or urine samples [4–8] or had inadequate sensitivity (LLOQ > 60 ng/mL) [9–12], which prevented us from adopting these methods for routine clinical analysis. Taylor *et al.* [14] reported an LC-MS/MS method for indomethacin coupled with solid-phase extraction (SPE) which achieved good sensitivity (LLOQ=5 ng/mL) and smaller volume of plasma (0.1 mL). However, the extraction recovery with this method was only 74% [14]. In addition, the SPE method is time-consuming and expensive considering the large number of patient samples in clinical trials. On the other hand, a liquid-liquid extraction method achieved a higher extraction recovery of indomethacin from human plasma (95%) [7].

Therefore, the goal of the current investigation was to develop and validate a sensitive, selective and rapid LC-MS method coupled with simple liquid-liquid extraction for quantitative determination of indomethacin in plasma and urine samples of pregnant patients under treatment.

2 Experimental

2.1 Chemicals and reagents

Chemicals were purchased from the following companies: indomethacin and chloroform from Sigma Chemical Co. (St. Louis, MO); *d*₄-indomethacin from Toronto Research Chemicals Inc. (North York, Canada); HPLC-grade acetonitrile, methanol, formic acid and hydrochloric acid from Fisher Scientific (Fair Lawn, NJ).

2.2 Biological samples

Pooled blank human plasma containing heparin sodium as the anticoagulant was purchased from Innovative Research Inc. (Novi, MI). The urine samples and individual plasma samples used as blanks were donated by colleagues. Maternal plasma and urine samples were obtained from pregnant patients enrolled in a pilot PK study of indomethacin according to a protocol approved by the Institutional Review Board of The University of Texas Medical Branch at Galveston. The blood samples were collected into BD Vacutainer® tubes containing heparin lithium and the plasma was separated by centrifugation. Urine samples were collected at intervals and the volume for each was recorded. All biological samples were kept frozen at –70°C until analysis.

2.3 LC-MS conditions

LC-MS instrument consisted of a Waters 600E multi-solvent delivery system, 717 autosampler and a Waters EMD 1000 single-quadrupole mass spectrometer (Milford, MA) controlled by Empower™ 2 software (Waters, Milford, MA). Indomethacin was separated on a Waters Symmetry C₁₈ column (150 × 4.6 mm, 5 µm) fitted with a Phenomenex C₁₈ guard column (4 × 3.0 mm). The mobile phase consisted of 0.05% (v/v) formic acid aqueous solution and acetonitrile (47:53, v/v). Isocratic elution was performed for 11 min at a flow rate of 1.0 mL/min. The eluent from the HPLC column was split at 4:1 ratio and directed to the mass spectrometer at a flow rate of 200 µL/min.

The mass spectrometer was equipped with an electrospray ion source (ESI) and operated in positive mode. MS parameters were as follows: capillary voltage, 4.0 kV; cone voltage, 55 V; source temperature, 95°C; desolvation temperature, 200°C; desolvation gas flow rate, 150 L/h; cone gas flow rate, 150 L/h. Indomethacin and *d*₄-indomethacin (internal standard, IS) were monitored by selected ion monitoring (SIM) at *m/z* 139 for indomethacin, and *m/z* 143 for the IS.

2.4 Preparation of stock and working standard solutions, calibration standards and quality control (QC) samples

The stock solutions of indomethacin and its internal standard were prepared in 30% (v/v) methanol. Working standard solutions of Indomethacin were prepared in the range of 0.148 to 29.7 µg/mL for the analysis of maternal plasma samples, and 0.105 to 42.1 µg/mL for urine samples. The final concentration of the IS working solution was 2.00 µg/mL for analysis of plasma and urine samples. These solutions were stored at 4°C.

The calibration standards were prepared by adding 20 µL of working standard solution into 200 µL of either plasma or urine to achieve a final indomethacin concentration range of 14.8 to 2.97×10³ ng/mL for plasma samples and 10.5 to 4.21×10³ ng/mL for urine samples. Quality control (QC) samples were prepared using blank plasma or urine at high, medium, and low concentrations as well as at the lower limit of quantification (LLOQ) of indomethacin.

2.5 Preparation of plasma and urine samples

An aliquot of the IS working solution (20 µL) was added to 200 µL of untested patient samples (plasma or urine), calibration standards or QC samples. The mixture was vortexed for 30 sec, then 50 µL of 1 M hydrochloride acid and 900 µL of chloroform were added and vortexed for 3 min. The solution was centrifuged at 12,000 × *g* for 5 min, and the organic layer transferred to a clean tube. The remaining aqueous layer was re-extracted with 900 µL of chloroform as described above. The two organic layers were combined and dried under a stream of air at 40°C. The dry residues were reconstituted in 100 µL of the mobile phase and centrifuged at 12,000 × *g* for 15 min. An aliquot (80 µL) of the supernatant from each sample was injected into the HPLC system.

2.6 Method validation

The method was validated according to the guidelines issued by the FDA for bio-analytical method validation [15]. The selectivity was determined by analyzing pooled and individual blank plasma and urine samples from six individual donors. The calibration curves were fitted using weighted least-squares linear regression of the internal ratio (peak area of the analyte/peak area of the IS) versus concentration. The limit of detection (LOD) was determined at a signal-to-noise (S/N) ratio of 3:1 by comparing test results from samples with known concentrations of indomethacin to blank samples. The lower limit of quantification (LLOQ) was defined as the lowest concentration that produced an S/N >10 and could be quantified with an RSD < 20% and accuracy in the range of 80–120% [15].

The extraction recovery of indomethacin from plasma and urine samples was evaluated by comparing the peak area of indomethacin in QC samples to its peak area in the post-extracted samples at high, medium and low concentrations. The matrix effect of indomethacin and the IS was evaluated quantitatively by calculating the Matrix Factor, which is defined as the ratio of the analyte peak area of post-extraction samples to the analyte peak area of pure standards [16].

Intra-day and inter-day accuracy and precision of indomethacin in plasma and urine samples were evaluated by the analysis of six replicate QC samples at high, medium and low concentrations. For the intra-day assay accuracy and precision, QC samples were analyzed using a calibration curve prepared on the same day; for the inter-day assay accuracy and precision, the QC samples were analyzed on three consecutive days. Accuracy was expressed as [mean of measured concentration/nominal concentration] $\times$ 100% and the precision was represented by the relative standard deviation (RSD).

The stability of indomethacin in human plasma and urine samples was assessed by analyzing replicate QC samples at high and low concentrations. Freeze-thaw stability was determined after three freeze-thaw cycles. Each cycle consisted of freezing at -70°C for 12 h and then thawing under the room temperature ($22\text{--}25^{\circ}\text{C}$). For short-term stability and long-term stability investigations, the unprocessed QC samples were kept at room temperature for 4 h and at -70°C for 30 days, respectively. Then these QC samples were processed and analyzed. The concentration of indomethacin was calculated using a freshly prepared calibration curve.

3 Results and Discussion

3.1 Method development

3.1.1 Mass spectrometric conditions—The ionization of indomethacin was investigated with the ESI interface in both positive and negative ion modes. The positive ion mode was chosen for analysis of indomethacin because of higher sensitivity of all its fragmentation ions than the negative ion mode. The quasi-molecular ion $[\text{M}+\text{H}]^{+}$ of indomethacin at m/z 358 could not be observed at low cone voltage most likely due to the weak amide bond of indomethacin that fragments easily into two ions at m/z 139 and m/z 174 (Fig. 1.). Increasing the cone voltage from 15 V to 75 V resulted in the formation of the fragmentation ions due to in-source fragmentation. The ion at m/z 139 was the major fragmentation ion when the cone voltage was less than 55 V, whereas the ion at m/z 111 was the major fragmentation ion when the cone voltage was increased to 75 V. The fragmentation ion at m/z 174 was excluded from further consideration because it is identical for both of indomethacin and its internal standard. An interfering peak from the extracted individual plasma and urine samples was observed in the SIM channel at m/z 111, whereas the m/z 139 channel was clean. Therefore, the channel at m/z 139 was finally chosen as the SIM monitoring ion and the cone voltage of this channel was optimized at 55 V to achieve the highest sensitivity. The deuterium labeled indomethacin (d_4 -indomethacin) was chosen as the internal standard to minimize the influence of matrix effect on indomethacin [16]. The MS conditions of d_4 -indomethacin were similar to the MS conditions of its non-labeled isotope and the SIM monitoring ion was setup at m/z 143.

3.1.2 Liquid Chromatographic conditions—Adequate chromatographic separation of indomethacin from the co-eluted endogenous compounds was necessary to avoid the matrix effect in the extracted plasma and urine samples [17]. In addition, to achieve the same degree of matrix effect, the HPLC conditions were optimized to elute indomethacin and its IS at the same retention time [18].

The composition of the mobile phase, organic modifiers, buffer and pH value were screened. Acetonitrile was chosen as the organic modifier because indomethacin and d_4 -isotope tend to be separated in the methanol system due to the deuterium isotope effect. The aqueous solution was tested using ammonium acetate buffer at different pH values (10 mM, pH adjusted by acetic acid) or different percentages of formic or acetic acid (0.5%, 0.1% and 0.05% (v/v)). Adequate chromatographic separation of indomethacin from the co-eluted interfering peaks was not achieved using either ammonium acetate or acetic acid. Thus, the

final composition of the mobile phase was 53% acetonitrile and 47% formic acid (0.05%, v/v) in aqueous solution with isocratic elution at a flow rate of 1.0 mL/min.

3.1.3 Sample extraction—In previous reports, liquid-liquid extraction (LLE) [7, 12], solid phase extraction (SPE) [4–6, 14] and protein precipitation (PP) [8–11] were utilized for the extraction of indomethacin from various biological samples. The precipitation of protein in our plasma and urine samples by adding methanol or acetonitrile resulted in significant ion-suppression of indomethacin. For the optimization of the LLE method, pH additives (NaOH, NH₄OH, HCl, formic acid and acetic acid) and organic solvents (hexane, ethyl acetate and chloroform) were tested. However, under alkaline pH, the addition of organic solvents resulted in the formation of an emulsion in plasma samples. Under acidic conditions, chloroform exhibited good extraction recovery of indomethacin. The highest extraction recovery (>94%) of indomethacin was achieved by adding 50 µL of HCl aqueous solution (1.0 M) to 200 µL of plasma or urine samples, followed by extracting twice with chloroform.

3.2 Method validation

Fig. 2. shows typical SIM chromatograms of the blank samples, blank samples spiked with indomethacin and the IS, and samples collected from pregnant patients treated with indomethacin. The retention times of indomethacin and the IS in the chromatograms were 8.6 and 8.5 min, respectively. The areas of the endogenous peaks that co-eluted with indomethacin was < 5% of the peak area of indomethacin at LLOQ concentrations which indicated that no significant interfering peak was observed at the retention times of either indomethacin or IS.

The calibration curves for plasma and urine samples were fitted by weighted least-squares linear regression analysis of the internal ratio versus concentration (Table 1). The weighted factor for the calibration curves was selected as $1/x^2$ for both plasma and urine quantification according to the sum of percentage relative error of each calibration point [19]. The calibration curve exhibited good linear regression within the tested ranges of 14.8 to 2.97×10^3 ng/mL ($r^2 > 0.99$) for plasma and 10.5 to 4.21×10^3 ($r^2 > 0.98$) for urine samples, respectively. The lack of fit test of the regression between the concentration and the internal ratio was not significant as indicated by the F -tests at $\alpha = 0.05$ levels [20]. The LLOQ was set as the lowest concentration of the calibration curves (Table 1), and inter- and intra-day accuracy values at the LLOQ were in the range of 86% to 106% with precision < 16% (Table 2). The LOD concentrations of plasma and urine samples were 3.71 ng/mL and 2.63 ng/mL, respectively.

The extraction recovery of indomethacin from plasma and urine samples ranged between 94% and 104% with variation < 11% at high, medium and low concentrations. The Matrix Factor of indomethacin in six individual samples from plasma and urine at high, medium and low concentrations ranged between 76% and 85%. Although the above results (data not shown) indicate that there was moderate ion suppression of indomethacin in plasma and urine matrices, the variability of the matrix factor was < 5% and < 9% across six individual plasma and urine samples, respectively.

The intra-day accuracy of the method ranged between 90% and 108% with precision < 6%, and the inter-day accuracy ranged between 94% and 107% with precision < 8% for plasma and urine QC samples at high, medium and low concentrations. These results indicate acceptable reproducibility of the method used.

The stability of indomethacin in plasma and urine samples was investigated under a variety of storage and handling conditions which exceeded the routine sample preparation and

storage time. The recovery of indomethacin in the freeze-thaw, short-term and long-term stability samples ranged between 92% and 113% with precision < 10%, indicating indomethacin to be stable in the above storage conditions.

3.3 Application of the method

The validated method was utilized to support a clinical trial of indomethacin in pregnant patients under the treatment of 25mg indomethacin every 6 h to determine its pharmacokinetics during pregnancy. After reaching steady-state, maternal vein blood and urine samples were collected at predetermined time points. Freshly prepared QC samples at high, medium and low concentrations were determined daily together with the clinical samples.

To date, total of 243 plasma samples and 124 urine samples from 31 patients were analyzed. The concentrations of indomethacin ranged between 30.0 ng/mL and 2.26×10^3 ng/mL in plasma samples and between 26.5 ng/mL and 3.92×10^3 ng/mL in urine samples. In one patient, the plasma concentration of indomethacin was lower than LOD, whereas the urine concentration in the same patient ranged between 364 ng/mL and 1.11×10^3 ng/mL. Despite the wide range of indomethacin concentrations between individual samples, it is important to note that the patient sample concentrations fit within the linear range of this validated method.

4. Conclusion

An HPLC with single quadrupole mass spectrometry method was developed to determine the concentration of indomethacin in plasma and urine samples obtained from pregnant patients under treatment of indomethacin. The method is based on simple liquid-liquid extraction and isocratic chromatography and provided adequate sensitivity, selectivity, accuracy and precision. This method is suitable for determining the pharmacokinetic parameters of indomethacin during pregnancy.

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Highlight of the manuscript

- The LC-MS quantitative method of indomethacin was developed and validated in human maternal plasma and urine samples.
- Selectivity, sensitivity and reproducibility were achieved by using an in-source fragmentation technique.
- The lower limit of quantification of indomethacin is less than 14.8ng/mL in maternal plasma and 10.5ng/mL in human urine samples.
- The validated method has been used to determine indomethacin concentrations in samples of plasma and urine from pregnant patients.

Fig. 1.
Electrospray mass spectra and the proposed fragmentation pattern of indomethacin.

Fig. 2.

Selected ion monitoring (SIM) chromatograms for the determination of indomethacin in maternal plasma (A1–A3) and urine (B1–B3):

(A1) chromatogram of a blank plasma sample; (A2) chromatogram of a blank patient plasma sample spiked with indomethacin (24.8 ng/mL); (A3) plasma sample of pregnant patient following oral administration of an indomethacin dose of 25 mg (q.i.d.); (B1) chromatogram of a blank urine sample; (B2) chromatogram of a blank urine sample spiked with indomethacin at a concentration of 35.1 ng/mL; (B3) urine sample of a pregnant patient following administration of an indomethacin dose of 25 mg (q.i.d.). INDO was denoted as indomethacin.

Table 1

Calibration curves for indomethacin in maternal plasma and urine samples (n=3).

Biological samples	Regression Equation ^a	Weighting Factor	r^2	F -test ^b		Linear Range (ng/mL)	LOD (ng/mL)
				F	p -value		
Maternal plasma	$y=0.0042+0.0050x$	$1/x^2$	0.993	1.65	0.202	$14.8-2.97 \times 10^3$	3.71
Maternal urine	$y=0.0099+0.0056x$	$1/x^2$	0.983	0.92	0.515	$10.5-4.21 \times 10^3$	2.63

^aIn the regression equation $y = b + ax$, x refers to the concentration (ng/mL) of indomethacin in biological samples, and y refers to the peak area ratio of indomethacin versus the IS.

^b F -test for lack-of-fit.

Table 2

Intra-day and inter-day precision and accuracy of the method for maternal plasma and urine samples.

Specimen	Nominal Concentration (ng/mL)	Intra-day (n=6)				Inter-day (n=3)			
		Mean Measured Concentration (ng/mL)	Mean Accuracy ^a (%)	Precision (RSD, %)	Mean Measured Concentration (ng/mL)	Mean Accuracy ^a (%)	Precision (RSD, %)	Mean Measured Concentration (ng/mL)	Precision (RSD, %)
Maternal plasma	14.8	15.0	101	4.4	15.7	106	6.3		
	49.5	48.1	97	2.9	50.6	102	5.3		
	1.48×10 ³	1.40×10 ³	94	1.4	1.45×10 ³	97	3.6		
Maternal urine	2.97×10 ³	3.07×10 ³	103	1.1	3.13×10 ³	105	5.3		
	10.5	9.04	86	15.8	9.95	94	12.9		
	21.1	18.7	90	5.4	19.8	94	7.1		
	2.11×10 ³	1.98×10 ³	94	0.7	2.00×10 ³	95	2.0		
	4.21×10 ³	4.53×10 ³	108	1.0	4.50×10 ³	107	3.4		

^a accuracy (%) = (mean measured concentration/nominal concentration)×100%.

Table 3

Stability of indomethacin in maternal plasma and urine samples (n=3).

Biological samples	Nominal Concentration (ng/mL)	Freeze and thaw stability (3 cycles, -70°C)			Short-term stability (4h, 22-25°C)			Long-term stability (30 days, -70°C)		
		Mean Measured Concentration (ng/mL)	Mean Accuracy ^a (%)	Precision (RSD, %)	Mean Measured Concentration (ng/mL)	Mean Accuracy ^a (%)	Precision (RSD, %)	Mean Measured Concentration (ng/mL)	Mean Accuracy ^a (%)	Precision (RSD, %)
Maternal plasma	49.5	51.2	103	2.4	49.1	99	7.1	54.3	110	4.4
	2.97×10 ³	3.36×10 ³	113	5.7	3.11×10 ³	105	3.9	3.26×10 ³	110	0.6
Maternal Urine	21.1	19.5	92	9.6	19.9	92	7.6	20.7	98	3.9
	4.21×10 ³	4.75×10 ³	113	2.4	4.21×10 ³	100	4.7	3.94×10 ³	94	1.1

^a accuracy (%) = (mean measured concentration/nominal concentration)×100%.