High-Performance Liquid Chromatographic Assay of Indomethacin in Porcine Plasma with Applicability to Human Levels

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Abstract

A high-performance liquid chromatography (HPLC) assay is described for the determination of indomethacin in porcine plasma using acetonitrile to precipitate plasma proteins and for the one-step extraction. Calibration curves (using the internal standard method) are linear (r^2 > 0.98) over the concentration range of 50.0 to 3000 ng/mL in both mobile phase and plasma. Precision, expressed as the inter- and intraday coefficient of variation (*n* = 5), is < 7% on the same day and < 5% between days at each plasma control sample of 300, 1000, and 3000 ng/mL, respectively. System precision, calculated as the coefficient of variation (*n* = 5), is < 7% at 3000 ng/mL of indomethacin, and the limit of quantitation in plasma is 50 ng/mL. The absolute recovery for both indomethacin and the internal standard (mefenamic acid) from plasma is over 97% (*n* = 3), and the concentrations do not deviate more than –2.9% to 2.4% from their actual values. The specificity of the method is confirmed. This technique is thus reported to be both rapid and specific. The real advantage is the small sample volume required (500 µL), which allows it to be considered for the quantitation of indomethacin in plasma from paediatric patients.

Introduction

Indomethacin, a nonsteroidal anti-inflammatory drug (NSAID) with predominantly cyclo-oxygenase isoform selectivity, is highly protein bound, resulting in a lack of information regarding the concentration-effect profile of indomethacin. The high degree of protein binding results in high interpatient variation in the fraction of the unbound NSAID and the plasma-bound NSAID concentration (1). This interpatient variation in NSAID concentration has resulted in many animal studies employing a pharmacological dose, rather than a therapeutic dose, for an acute period. The risks of side effects associated with this high dose administered for a therapeutic time period thus limits the application.

A number of studies have reported on the quantitation of indomethacin levels in serum or plasma (or both) in rats, horses, and humans (2–5). However, no method currently exists for its quantitation in pigs. These methods have mostly used liquid–liquid extraction with organic solvents after acidification of the plasma to precipitate proteins, followed by evaporation of the organic supernatant. This generic method, has been conducted using various organic solvents including dichloromethane, petroleum ether, methylene chloride, percholic acid, ethyl acetate, and acetonitrile (3–7), though several methods have reported acidification of the sample using citrate buffer, phosphate buffer, and hydrochloric acid to improve extraction efficiency (4,7). Alternatively, there are some studies in which cartridges have been used to purify samples (2,8). These methods produce clean samples but are both expensive and time consuming.

This paper describes an automated technique adapted from the previously described methodologies (3,4), utilizing a one-step liquid–liquid extraction procedure without the requirement of sample evaporation or sample resuspension. In this study, the drug recovery rate was reported, method was validated, and reproducibility was confirmed. The high-performance liquid chromatography (HPLC) method developed allowed for the rapid analysis of indomethacin in porcine plasma by utilizing easy extraction procedures and simple, cost-effective chromatographic conditions while maintaining validity. The method can be easily adapted for human pharmacokinetic studies. The validation range of this method reflects the therapeutic drug range expected in humans (9).

Experimental

Reagents

All chemicals used were reagent grade. Acetonitrile and methanol were HPLC grade (EM industries Inc, Hillsborough, NJ). Water for chromatography was obtained using a Milli-Q (Millipore, Billerica, MA) water purification system. The internal standard (mefenamic acid) was obtained from Sigma (Brisbane, Australia), and the analytical standard

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(indomethacin) was generously donated by AlphaPharm Ltd. (Brisbane, Australia).

Chromatographic conditions

The HPLC system consisted of a Prostar 240 solvent pump fitted with a Prostar 410 autosampler with a 100-µL sample loop and a Prostar 330 UV–vis detector (Varian, Palo Alto, CA) coupled with a Star Workstation data integrator and recorder (Varian Analytical Instruments, Walnut Creek, CA). Chromatographic separation was achieved on a Res Elut reversed-phase column (4.6 × 150 mm, C18, 5 µm, 90Å) (Varian Analytical Instruments). The mobile phase consisted of 60% acetonitrile in 0.02M sodium acetate buffer and was adjusted to pH 3.6 using orthophosphoric acid. Prior to use, the mobile phase was filtered (0.2 µm) and degassed for 20 min. The flow rate was 1.0 mL/min, with a backpressure of 120 atmospheres, and the detector wavelength was set at 320 nm at a slit width of 4 nm. The concentrations of indomethacin and internal standard were determined by calculation of the area under the curve. All analyses were performed at room temperature (25°C ± 2°C).

Standards

A stock solution of indomethacin (0.1 mg/mL) was prepared in methanol. Stock solutions were stored at –20°C and were considered stable for up to 30 days as reported by Curry et al. (10). Standard solutions of 0.01 and 0.001 mg/mL were prepared by diluting aliquots of the stock solutions in methanol daily. The same procedure was utilized for the internal standard, mefenamic acid (0.1 mg/mL).

Both mobile phase and plasma calibration standards were prepared by adding an appropriate volume of standard solutions of indomethacin and internal standard, corresponding to the concentrations of 0, 10, 50, 300, 500, 1000, 1500, and 3000 ng/mL, into either blank porcine plasma obtained from healthy Large White pigs (Tony's Piggery, Charter's Towers, Australia) (~ 4 months, 60 kg) or freshly prepared mobile phase and processed immediately.

Sample preparation

All porcine blood samples were collected from the jugular vein using a 1.5-inch sterile 18-gauge needle and a sterile disposable syringe. Samples were collected into 1-mL paediatric tubes with tri-potassium edetic acid (Greiner bio-one, Tokyo, Japan). Samples were centrifuged immediately at 1000 × *g* for 4 min. The plasma was then transferred into a 1.5-mL lock capped microcentrifuge tube (Sarstedt, Technology Park, South Australia, Australia) for extraction.

Extraction procedure

Five hundred microliters of porcine plasma standard or porcine plasma sample was dispensed into 1.5-mL Eppendorf centrifuge tubes (Eppendorf, Westbury, NJ). Five hundred microliters of acetonitrile was added to the plasma to precipitate proteins and as the extraction solvent. The samples were then agitated on a high-speed vortex for 2 min and further mixed using a rotary shaker for 2 min. The precipitated proteins were then pelleted by centrifugation at 10,000 × *g* for 10 min (Heraeus Sepateh, Radiometer Pacific, Brisbane, Australia). The supernatant was transferred to a clean lock-capped 1.5-mL microcentrifuge tube and a further 100 µL of acetonitrile was added. This supernatant was introduced into an HPLC vial, and 100 µL was injected onto the column using the autosampler.

Calibration, accuracy, and precision

Quantitation is based on calibration curves constructed using the peak-area ratio of indomethacin to internal standard versus the actual concentration of indomethacin. Calibration curves were constructed in mobile phase and plasma with concentrations of 0, 50, 300, 500, 1000, 1500, and 3000 ng/mL. Each sample was analyzed in triplicate, and the regression lines were generated over 5 consecutive days using the method of least squares. The linearity of the method was assessed by construction of response factor plots.

Intraday reproducibility was tested using three different control samples with low, medium, and high levels of indomethacin for both the plasma and mobile phase (300, 1000, and 3000 ng/mL). Each sample was analyzed in triplicate. The procedure was repeated on 3 consecutive days to allow for the determination of interday precision and accuracy. Accuracy was estimated using the mean percentage error, based upon differences between actual and predicted concentrations. The precision, expressed as a percentage, was evaluated by calculating the intra- and interday coefficients of variation.

Limits of detection and quantitation

The limit of detection (LOD) is a parameter that provides the lowest concentration in a sample that can be detected from background noise but not quantitated. LOD was determined using the signal-to-noise ratio (s/n) of 3:1 by comparing test results from samples with known concentrations of analytes with blank samples.

The limit of quantitation (LOQ) is defined as the lowest concentration of analyte that can be determined with acceptable precision and accuracy. The LOQ was found by analyzing a set of mobile phase and plasma standards with a known concentration of indomethacin.

Recovery and specificity

The extraction efficiency was determined by comparing the peak area of known amounts of indomethacin and internal standard (mefenamic acid) in mobile phase (unextracted) with the peak area of samples containing the same amounts of indomethacin and internal standard in plasma (after extraction). The recovery was determined at 300 ng/mL (n = 3), 1000 ng/mL (n = 3), and 3000 ng/mL (n = 3) of indomethacin.

The specificity of the method was determined by comparing the chromatograms obtained from the plasma samples containing indomethacin and mefenamic acid with those obtained from blank plasma samples and analyzing them for peaks interfering with the detection of indomethacin or the internal standard. The spectral purity parameters for the analytes were stored in a spectral library. Subsequent peaks were tested to determine the level of purity compared to the stored spectra in order to confirm specificity.

Animal study

To assess the ability of the assay to measure indomethacin concentrations for in vivo samples, a preliminary pharmacokinetic trial was conducted in a female Large White pig (six weeks old, weight 10.0 kg). The animal received a single 30-mg (3 mg/kg) oral dose of indomethacin in a capsule. Serial blood samples were collected from the jugular vein at 0, 2, 4, 6, 8, and 24 h post-ingestion, and the resultant plasma samples were assayed immediately, as described previously. The area under the plasma concentration versus time curve from time of dosing to infinity ($AUC_{0-\infty}$) was determined using the log-linear trapezoidal rule.

Results and Discussion

Figure 1 shows typical chromatograms of blank (drug free) plasma (A), blank plasma spiked with indomethacin corresponding to a concentration of 3000 ng/mL (B), and the internal standard and a porcine plasma sample 6 h after oral administration of 3 mg/kg of indomethacin (C). The retention times are 3.6 and 5.3 min for indomethacin and the internal standard, respectively, with a total run time of less than 7 min.

The concentration of indomethacin calculated from the integration area under the peaks was linearly related to the internal standard over the concentration range of 50–3000 ng/mL based on calibration curves, with a correlation coefficient of > 0.98. The use of a weighted least squares regression resulted in a less than 10% deviation between the actual standard concentration and the experimentally determined standard concentration calculated from the regression equation. By constructing response factor plots, the linearity of the method was shown as the plots followed horizontal behavior in each matrix (acetonitrile and plasma). The intra- and interday precision and accuracy studies are shown in Table I. Based on this data, the validated LOQ of the method was defined as the concentration of analyte that produced a s/n of 10:1; 50 ng/mL was accepted based on 500 µL of porcine plasma. The LOD was determined to be 10 ng/mL based on a s/n of 3:1.

The mean extraction efficiencies of indomethacin from plasma at concentrations of 300, 1000, and 3000 ng/mL (n = 3) were 98.6%, 99.6%, and 99.1%, respectively. Spectral purity parameters obtained by photodiode array at the start and end of the calibration indicated that the peaks obtained for indomethacin and internal standard were pure, and no degradation was detected.

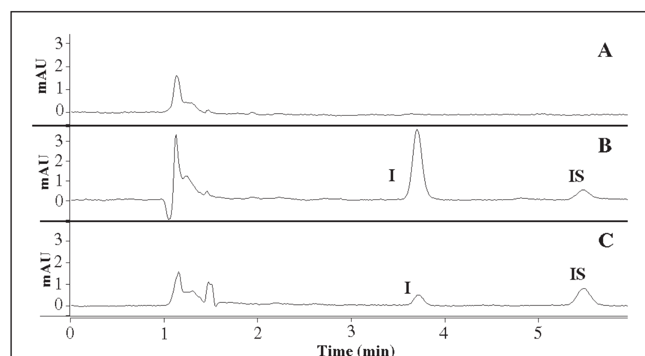

Figure 1. Chromatograms derived from assay of blank porcine plasma (A), porcine plasma spiked with 300 ng/mL of indomethacin (I) (B), and porcine plasma 6 h after oral administration of 3 mg/kg indomethacin (C), with mefenamic acid as the internal standard (IS).

In the pig given a 30-mg oral dose of indomethacin, plasma samples were analyzed using the described HPLC method. In this animal, the maximum indomethacin plasma concentration was 122 ng/mL at approximately 6 h post-dose (Figure 2). The area under the plasma concentration-time curve ($AUC_{0-\infty}$) indicated a concentration of 6.62 mg h/L. Although the dose rate administered was based on a human regimen, when a dose of 3 mg/kg/dose was administered three times a day, it proved to be fatal to pigs on a long-term basis (results not shown).

Acetonitrile was used for denaturing of plasma proteins and also as the solvent. Attempts to acidify the plasma sample with 500 µL of citrate buffer (and thus denature plasma proteins) and the use of dichloromethane, petroleum ether (or 50:50, v/v, % mixture), or ethyl acetate as extraction solvents failed because the method was not reproducible, and the extraction efficiency was less than 60%. The low extraction efficiency observed was because of inadequate denaturing of plasma proteins because

Table I. Validation Data for the Assay of Indomethacin in Porcine Plasma

Indomethacin concentration (ng/mL)				
Added	Experimental (mean ± SD)	Coefficent of variation (%)	Recovery (%)*	Accuracy (%)†
Interday precision (n = 3)				
300	294.9 (± 18.8)	6.33	98.3	–1.7
1000	1024 (± 34.82)	3.4	102.4	+2.4
3000	2943 (± 122.49)	4.12	98.1	–1.9
Intraday precision (n = 3)				
300	296.4 (± 12.64)	4.4	98.8	–1.2
1000	971 (± 22.9)	2.08	97.1	–2.9
3000	3000 (± 114.4)	3.8	100.0	0

* Recovery is defined as the measured/spiked × 100%.
† Accuracy is defined as the mean percentage error = (measured – spiked)/spiked × 100%.

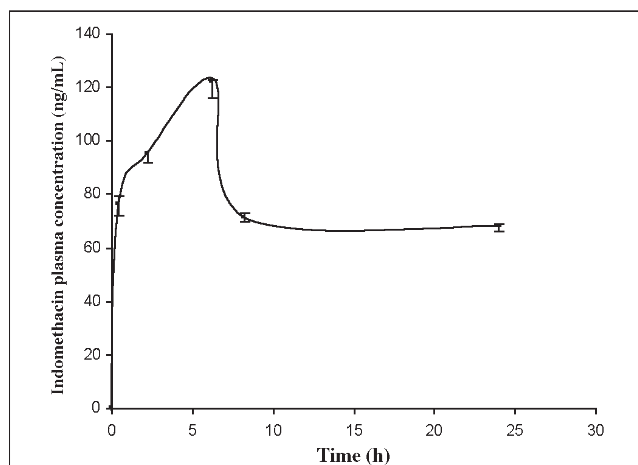

Figure 2. Plasma indomethacin concentration versus time curves for 10.0 kg pig dosed with 30 mg of oral indomethacin.

indomethacin is extensively plasma protein bound and because of the loss of drug during the evaporation of the organic extraction solvent. Therefore, acetonitrile was employed for both purposes, which allowed for the supernatant to be directly injected onto the column. Some authors have criticized the use of acetonitrile because plasma proteins are inadequately precipitated. However, studies were conducted with various volumes of acetonitrile to ensure that complete protein precipitation was achieved; otherwise there is a risk of blocking the chromatography column and shortening column life. When the initial volume of acetonitrile was 500 µL or greater, no further protein precipitation was observed with any subsequent additions of solvent to the supernatant. Protein precipitation was determined by agitation of the sample and centrifugation, as previously stated, to determine if a protein pellet had formed. Furthermore, the extraction efficiency of over 97% for the predicted concentration range for indomethacin validates this method of protein precipitation.

Conclusion

This method has been developed and validated for the determination of indomethacin in porcine plasma across the therapeutic range used in animals and humans. Unlike most liquid–liquid extraction methods, which require the extraction solvent to be evaporated and the samples reconstituted prior to injection into the system, a one-step extraction phase that is rapid and simple is utilized. A major advantage of this method is that the analyte and internal standard can be eluted into a small volume of acetonitrile, which was then directly injected onto the HPLC system, allowing for detection of low concentrations of indomethacin. The quantitation level of 50 ng/mL was considered acceptable because expected therapeutic plasma levels were above this value. Specifically, a therapeutic indomethacin plasma level required to achieve closure of the ductus arteriosus in preterm infants is 600 ng/mL, well within the validation range of this assay (11). Therefore, following validation of this method for human sampling, this HPLC method for the quantitation of indomethacin in plasma may have widespread application for routine drug assay laboratories, including the processing of paediatric samples. Additionally, all samples for this study were collected into 1-mL paediatric collection tubes. This volume of blood (500 µL of plasma) is routinely collected for pharmacokinetic and pharmacodynamic studies in children (12). This method is rapid, with the time required for the extraction and analysis of 20 samples being less than 3 h. Additionally, the reported extraction efficiency for other indomethacin assays is at greatest 90% (3,4), and the recovery rate for this method is greater than 97%, with a coefficient of variation of less than 7%. This extraction, detection, and quantitation of indomethacin by HPLC has been validated for pigs, providing investigators with rapid drug levels in this commonly utilized experimental animal.

This study describes the rapid and specific determination of indomethacin in porcine plasma samples and has the potential for use in determining indomethacin drug levels in patient blood samples for therapeutic drug monitoring, with application to the routine determination of indomethacin concentrations in paediatric patients.

References

1. J.H. Lin, D.M. Cocchetto, and D.E. Duggan. Protein binding as a primary determinant of the clinical pharmacokinetic properties of nonsteroidal anti-inflammatory drugs. *Clin. Pharmacokinet.* **12(6):** 402–32 (1987).
2. R.W. Berninger, D.A. Darsh, and D.R. Fulton. The use of reversed phase cartridges (C18) in processing plasma analysis of indomethacin by high-performance liquid chromatography. *J. Clin. Chem. Clin. Biochem.* **24(4):** 227–32 (1986).
3. Y.L. Brown, R.J. Kandrotas, J.B. Douglas, and P. Gal. High-performance liquid chromatographic determination of indomethacin serum concentrations. *J. Chromatogr.* **459:** 275–79 (1988).
4. A.G. Johnson and J.E. Ray. Improved high-performance liquid chromatographic method for the determination of indomethacin in plasma. *Ther. Drug. Monit.* **14(1):** 61–65 (1992).
5. E. Grippa, L. Santini, G. Castellano, M.T. Gatto, M.G. Leone, and L. Saso. Simultaneous determination of hydrocortisone, dexamethasone, indomethacin, phenylbutazone and oxyphenbutazone in equine serum by high-performance liquid chromatography. *J. Chromatog. B Biomed. Sci. Appl.* **738(1):** 17–25 (2000).
6. S.G. Owen, M.S. Roberts, and W.T. Friesen. Rapid high-performance liquid chromatographic assay for the simultaneous analysis of nonsteroidal anti-inflammatory drugs in plasma. *J. Chromatogr.* **416(2):** 293–302 (1987).
7. E. Vinagre, C. Rodriguez, M.I. San Andres, J.C. Boggio, M.D. San Andres, and T. Encinas. Pharmacokinetics of indomethacin in sheep after intravenous and intramuscular administration. *J. Vet. Pharmacol. Ther.* **21(4):** 309–14 (1998).
8. P.H. Hubert and J. Crommen. Automatic determination of indomethacin in human plasma using liquid-solid extraction on disposable cartridges in combination with HPLC. *J. Liq. Chromatogr.* **13(19):** 3891–3907 (1990).
9. S.H. Curry, E.A. Brown, H. Kuck, and S. Cassin. Preparation and stability of indomethacin solutions. *Can. J. Physiol. Pharmacol.* **60(7):** 988–92 (1982).
10. J.G. Hardman and L.E. Limbird. *Goodman and Gilman's the Pharmacological Basis of Therapeutics*, 10th ed. The McGraw-Hill Companies, New York, NY, 2001, pp. 143–44.
11. T.F. Yeh, B. Achanti, H. Patel, and R.S. Pildes. Indomethacin therapy in premature infants with patent ductus arteriosus—determination of therapeutic plasma levels. *Dev. Pharmacol. Ther.* **12(4):** 169–78 (1989).
12. R.E. Kauffman and G.L. Kearns. Pharmacokinetic studies in paediatric patients. Clinical and ethical considerations. *Clin. Pharmacokinet.* **23(1):** 10–29 (1992).

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