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Journal of Chromatography B, 830 (2006) 364–367
JOURNAL OF CHROMATOGRAPHY B
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Short communication

A rapid and sensitive microscale HPLC method for the determination of indomethacin in plasma of premature neonates with patent ductus arteriousus

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Received 14 September 2005; accepted 11 November 2005
Available online 1 December 2005

Abstract

Indomethacin (IND) is the drug of choice for the closure of a patent ductus arteriosus (PDA) in neonates. This paper describes a simple, sensitive, accurate and precise microscale HPLC method suitable for the analysis of IND in plasma of premature neonates. Samples were prepared by plasma protein precipitation with acetonitrile containing the methyl ester of IND as the internal standard (IS). Chromatography was performed on a Hypersil C_{18} column. The mobile phase of methanol, water and orthophosphoric acid (70:29.5:0.5, v/v, respectively), was delivered at 1.5 mL/min and monitored at 270 nm. IND and the IS were eluted at 2.9 and 4.3 min, respectively. Calibrations were linear ($r > 0.999$) from 25 to 2500 μg/L. The inter- and intra-day assay imprecision was less than 4.3 % at 400–2000 μg/L, and less than 22.1% at 35 μg/L. Inaccuracy ranged from −6.0% to +1.0% from 35 to 2000 μg/L. The absolute recovery of IND over this range was 93.0–113.3%. The IS was stable for at least 36 h when added to plasma at ambient temperature. This method is suitable for pharmacokinetic studies of IND and has potential for monitoring therapy in infants with PDA when a target therapeutic range for IND has been validated.

Keywords: Indomethacin; Patent ductus arteriosus; Premature neonates; HPLC

1. Introduction

The ductus arteriosus is a fetal channel connecting the main pulmonary artery with the thoracic aorta before birth. The ductus normally closes under the influence of prostaglandins within 48 h of birth, but it may fail to close in some circumstances such as congenital abnormalities and hypoxia, and occurs in about four in five infants born before 28 weeks gestation. Treatment options include fluid restriction, diuresis, maintenance of good oxygenation, surgical ligation, and use of non-steroidal anti-inflammatory drugs (NSAIDs) by virtue of their vasoconstrictive action in the tissues of the ductus [1]. Of these, indomethacin (IND) has long been the drug of choice for treating PDA. Indomethacin is extensively bound to plasma proteins [2], and has wide intersubject variability in the plasma concentrations, half-life and therapeutic response in premature neonates [3–5]. Our interests lay in correlating dose and IND plasma concentrations with outcome in PDA and in estimating its population pharmacokinetics and absolute bioavailability in premature neonates.

Several HPLC methods have been reported for the analysis of IND in biological fluids, especially plasma [6–11]. However, most of these have significant limitations such as tedious sample preparation, long run times, and plasma sample volumes too large to be used for premature infants. Some authors have used drugs commonly prescribed in clinical practice as internal standards, which potentially compromises application to some patients. We presently report a simple and rapid HPLC method which uses only 25 μL of plasma, requires little sample workup, has a very short run time, and which utilizes an indomethacin analog not used clinically as the internal standard. This method has been used to accurately and precisely measure IND plasma concentrations occurring after standard intravenous

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doi:10.1016/j.jchromb.2005.11.025

and nasogastric doses in the prophylaxis and treatment of PDA.

2. Experimental

2.1. Reagents and chemicals

IND (Fig. 1) reference material (99.7%) was obtained from Alphapharm Pty Ltd. (Carole Parke, Brisbane, Qld, Australia). The methylester of IND (Fig. 1) was synthesized and purified as described previously [12]. Acetonitrile and methanol were HPLC grade (Merck Pty Limited, Kilsyth, Vic., Australia). Orthophosphoric acid was obtained from Ajax Chemicals (Auburn, NSW, Australia). Reagent grade water (Milli-Q, Millipore, North Ryde, NSW, Australia) was used throughout. Pooled blank plasma from volunteer donors was obtained from the pathology department blood bank (Mater Health Services, South Brisbane, Qld, Australia).

2.2. Chromatographic equipment and conditions

An 1100 series isocratic liquid chromatography system (Agilent Technologies, Forest Hill, Vic., Australia) consisting of an automatic sample injector, variable wavelength UV detector, and ChemStation software (version A06.03) was used for peak data processing. All analyses were conducted at 20 ± 3 °C. Separations were performed on a Hypersil ODS C_{18} column (5 μm particles, 125 mm × 4 mm i.d., Agilent Technologies). The mobile phase of methanol (70%,v/v), water (29.5%, v/v) and orthophosphoric acid (0.5%, v/v) was membrane filtered (Millipore, 0.45 μm pore size) and degassed under negative atmospheric pressure, then pumped at 1.5 mL/min. The eluent was monitored at 270 nm, the predetermined maximum wavelength for IND in the mobile phase. The total run time for each analysis was 4.7 min.

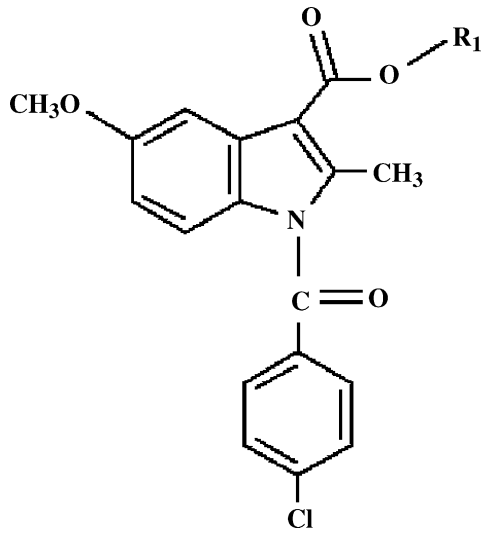

Fig. 1. Structure of indomethacin (IND; R_1 = H) and the internal standard (IS; R_1 = CH_3).

2.3. Stock solutions, plasma standards and controls

Separate stock solutions (1 g/L) of both of IND and IS were prepared in methanol. The IS stock was diluted to 400 μg/L with acetonitrile to obtain a working IS solution used in the samples workup. Blank plasma (1 mL) was supplemented from the IND stock to obtain a series of standards; 25, 50, 100, 250, 500, 1000 and 2500 μg/L. Likewise, IND seeded plasma controls of 35, 400 and 2000 μg/L were prepared independently from a fresh stock solution. The IND and IS stock solutions, and the IND plasma standards and controls were stored at −80 °C and they were stable for more than 6 months as determined by periodic analysis.

2.4. Sample preparation

A plasma standard, seeded control, or unknown sample (25 μL) was pipetted into a 1.5 mL polypropylene centrifuge tube to which was added 150 μL of working IS solution. The tube was vortex-mixed (30 s), then centrifuged (∼5000 × *g*, 2 min). The supernatant was carefully transferred to a 10 mL pyrex glass tube and the contents were evaporated to dryness at room temperature under a gentle nitrogen stream. The residue was dissolved in mobile phase (200 μL), and a 100 μL aliquot was injected.

2.5. Assay performance

The absolute recoveries of IND and the IS were calculated by comparing the peak heights of IND and IS in mobile phase with those obtained from the assay (n = 10) of 35, 400 and 2000 μg/L IND in plasma. The amount of IS added to each tube (60 ng) in the recovery assessment was constant. Calibration plots were constructed from the analysis of seven sets of IND standards (25, 50, 100, 250, 500, 1000 and 2500 μg/L) and the peak height ratio of IND to IS was regressed on IND concentration. The slope, y-intercept and coefficient of linear correlation (r) were estimated for each. A set of three seeded controls (35, 400, 2000 μg/L) was assayed four times on each of 5 consecutive days. IND concentrations were estimated on each occasion by inverse prediction from the calibration data constructed on each day. One-way analysis of variance (ANOVA) was used to calculate the intra-day and inter-day variability expressed as the imprecision (CV%), using all the data simultaneously [13].

3. Results and discussion

The chromatographic conditions were sufficient to resolve IND and the IS from endogenous plasma peaks. Fig. 2 shows representative chromatograms of blank plasma, plasma supplemented with 1000 μg/L of IND, and a plasma sample containing 438 μg/L drawn from 2-day-old neonate (29.5 weeks of gestational age, 1374 g birth weight) 3 h after administration of intravenous IND. The retention times for IND and the IS were 2.9 and 4.3 min, respectively. The mean (±S.D.) regression statistics for 6 calibration plots were; $y = (0.005 \pm 0.00001)\ x. + (0.015 \pm 0.009)$, where y is the peak height ratio and x is IND plasma concentration. The r was 0.999 ± .0006 (mean ± S.D.)

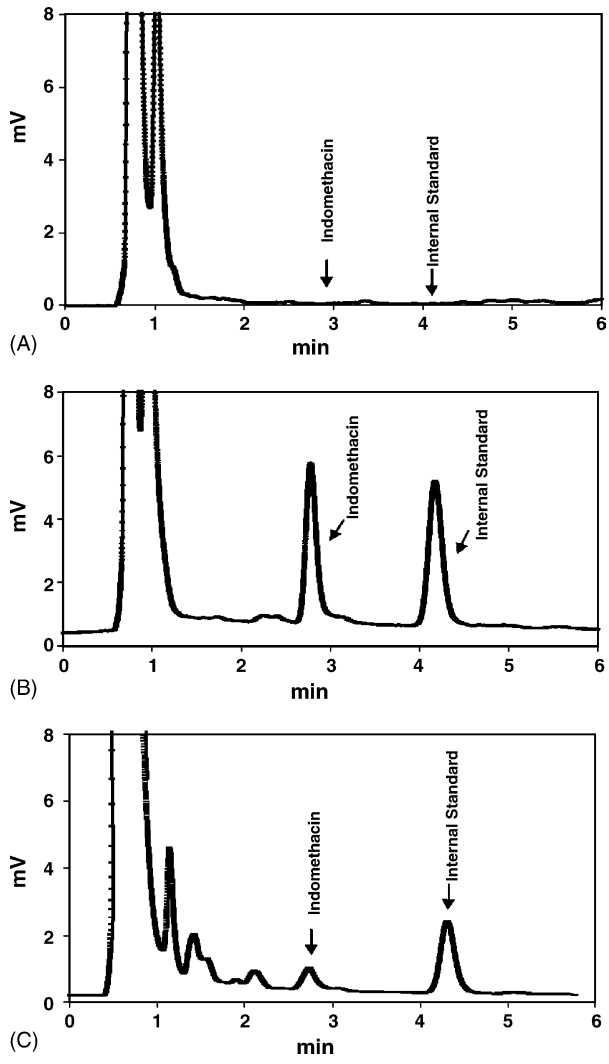

Fig. 2. Chromatograms of: (A) blank plasma; (B) IND plasma standard (1000 μg/L) and (C) plasma containing 438 μg/L drawn from a premature neonate 3 h after intravenous administration of IND (0.1 mg/kg).

indicating a linear calibration model was appropriate. The assay imprecision and inaccuracy are presented in Table 1. The lower limit of quantification (LOQ) was set a priori at 35 μg/L, the concentration at which the inaccuracy was less than 10% and the intra-day imprecision was <15% and which was sufficiently low to measure IND concentrations following administration to neonates from a previous study [4], and from our own patients (Fig. 3). While the inter-day imprecision of 22% ($n = 4$) at the LOQ was slightly greater than the Guidance for Industry recommended 20% limit, we expect that this would be reduced with an increased number of replicate assays at the LOQ. The limit

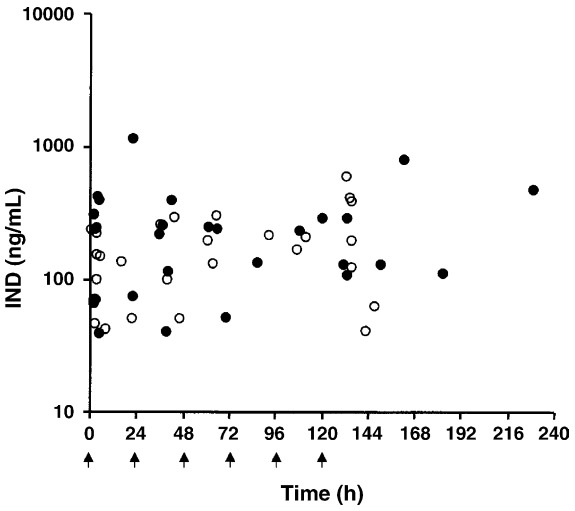

Fig. 3. Plasma concentration time data for premature neonates ($n = 20$) administered indomethacin (0.1 mg/kg) daily for 6 days, nasogastrically (open symbol) or intravenously (closed symbol). The arrows indicate the times at which an IND dose was given.

of detection (LOD) was 5 μg/L (350 ng on column), which was approximately three times baseline noise. The assay recovery results are presented in Table 2. The recovery of IND and the IS ranged from 93.0 to 113%, and 88.6 to 96.2%, respectively.

For the IS we used a compound that was not a drug used in the clinic, although this was not the case in a number of other studies [6–10]. The methylester of IND had similar chromatographic and spectral characteristics to IND and was eluted 1.4 min after it. The ethylester (Fig. 1, $R_1 = -CH_2-CH_3$) [12] was eluted at 5.8 min, but the peak shape was inferior to the IS. One potential disadvantage of carboxylic acid esters is hydrolysis to the acid by plasma esterases. However, the IS showed no evidence of decomposition over at least 36 h at 20 ± 3 °C when added to plasma at the concentration used in the assay, thereby obviating the need for addition of an esterase inhibitor such as paraxoan or sodium fluoride.

This method offers some substantial improvements over published assays. The plasma volume (25 μL) was much less than used previously [6–9,11] which made it especially suitable for studies in premature infants. The sample workup was simpler than in some previous studies [6–9,11], which, for example, used column switching [7] or liquid-liquid extraction with chloroform and ether [8]. The 4.7 min run time was shorter than for other methods [6–11], one of which required 20 min [10]. Accordingly, >50 unknown samples can be assayed in a day. A wavelength of 254 nm has been the most widely used mode of detection for IND [6–9]. With the mobile phase-column

Table 1
Inaccuracy and intra- and inter-day imprecision ($n = 4$)

Target (μg/L)	Mean ± S.D. (μg/L)	Inaccuracy (%)	Intra-day (CV, %)	Inter-day (CV, %)
35	32.9 ± 5.20	−6.0	13.9	22.0
400	403.9 ± 13.3	+1.0	4.0	2.80
2000	2004 ± 28.4	+0.2	3.2	4.30

Table 2
Absolute recovery of IND and IS

Concentration (μg/L)	Absolute recovery (%) (mean ± S.D., $n = 10$)	
	IND	IS
35	94.10 ± 2.42	88.57 ± 6.71
400	93.00 ± 3.67	93.51 ± 5.85
2000	113.3 ± 3.98	96.20 ± 9.39

combination described, greater sensitivity and less potential for endogenous interference was obtained using 270 nm. Sato et al. [10] used a wavelength of 205 nm, but their chromatograms showed endogenous peaks eluting very close to IND and the IS which may present problems with variation in mobile phase composition, and with column ageing. Furthermore, the LOD (50 ng/mL) was 10-fold greater than presently reported. We confirmed the selectivity of our method by noting a lack of interference from the most commonly used medications for neonates with PDA (theophylline, caffeine, amoxycillin, phenobarbital, gentamicin). We also evaluated fluorometric detection since IND has a methoxy group attached directly to a phenyl ring and, while potentially more selective, it was much less sensitive since IND had limited native fluorescence and offered no advantage over UV detection at 270 nm. To increase the fluorescence response for biological applications, others [11] have had to deacylate IND to deschlorobenzoyl-indomethacin (DBI), but apart from being time-consuming and more complex it offered no increase in sensitivity over our method and was only applicable to urine samples. To demonstrate the applicability of the method, Fig. 3 shows plasma data obtained from analysis of blood drawn from infants with PDA who were receiving IND by the nasogastric or intravenous routes, 0.1 mg/kg once daily, for 6 consecutive days. All samples contained concentrations of IND above the LOQ.

Acknowledgments

We thank Prof. L. Marnett (Center in Molecular Toxicology, Vanderbilt University School of Medicine, Nashville, TN, USA) for providing the indomethacin esters, and to Alphapharm Pty Ltd. (Carole Park, Qld, Australia) for providing the indomethacin reference material. We thank Professor D. Tudehope and Dr. P. Woodgate (Division of Neonatology, Mater Health Services, Brisbane, Qld, Australia) and Dr T. Donovan (Grantley Stable Neonatal Unit, Royal Brisbane Women's Hospital, Brisbane, Qld, Australia) for access to their patients. A Fellowship in Therapeutic Drug Monitoring provided to Dr. Dehghanzadeh by the Iranian Ministry of Health is gratefully acknowledged. We also acknowledge the provision of a Ph.D. scholarship to Dr. Al Za'abi by the Sultan Qaboos University (Muscat, Oman).

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