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Direct analysis of indomethacin in rat plasma using a column-switching high-performance liquid chromatographic system

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Abstract

We have established a robust, fully automated analytical method for the determination of indomethacin in rat plasma using a column-switching high-performance chromatographic system. The system consists of a precolumn and an analytical column connected in series via a switching valve. When a 50-μl portion of rat plasma containing a therapeutic level of indomethacin was applied directly to the system, the drug was automatically enriched in the precolumn (TSK BSA-ODS) by on-line solid-phase extraction. After elution of the plasma proteins, the analyte was automatically transferred to the analytical column (Zorbax Eclipse $\times DBC_{18}$) where chromatography was performed using isocratic elution and UV absorption detection at a wavelength of 254 nm. The separation mobile phase consisted of methanol–0.1% phosphoric acid (70:30, v/v) at a flow-rate of 1 ml/min. The calibration line for indomethacin showed good linearity in the range 50–10 000 ng/ml ($r > 0.999$) with the detection quantification of 50 ng/ml (RSD=2.6%). Accuracy ranged from −0.62 to 3.22%, and the within- and between-day precision of the assay was better than 6% across the calibration range. The analytical sensitivity and accuracy of this assay is suitable for characterization of the pharmacokinetics of topical administration of imdomethacin to rats. The method has been successfully used to provide pharmacokinetic data in a large number of diverse pharmaceutical studies. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Column switching; Indomethacin

1. Introduction

Indomethacin (IND) is a non-steroidal anti-inflammatory drug possessing anti-pyretic and analgesic properties [1,2]. Its anti-inflammatory effects stem from the inhibition of cyclooxygenase and resulting decrease in prostaglandin concentration in fluids and tissues [3]. IND is efficiently absorbed following oral administration, with plasma concentration peaking after 1–4 h; it is extensively bound to plasma proteins (90%) and has wide intersubject variability in its elimination half-life [4–6]. However, it has undesirable side effects such as gastrointestinal bleeding and exacerbation of renal inadequacy [7]. We have prepared several topical formulations of IND with the aim of eliminating these adverse effects, and we study their effects by evaluating drug plasma levels in rats.

Several methods have been reported for the determination of IND: thin-layer chromatography [8], gas chromatography [9,10], gas chromatographic–

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mass spectrometry [11], high-performance liquid chromatography (HPLC) [12–17] and HPLC–mass spectrometry [18]. However, these off-line procedures are unsuitable for use with large numbers of samples. The direct injection HPLC technique has many advantages, including easier sample preparation and automated analysis. The automated column-switching liquid chromatographic system has the advantage that the sample is directly injected into a column switching system in which the enrichment/clean-up step is performed by on-line liquid–solid extraction via a short precolumn, with complete exclusion of macromolecules and simultaneous extraction of low-molecular-mass analytes. With this automated column-switching technique the drug level in plasma is easily determined using a UV detector, with no loss of accuracy or sensitivity.

Accordingly, we have developed a simple, reproducible automated method for the determination of IND using an automated column-switching liquid chromatographic system. This method applies the integrated sample clean-up configuration, using a precolumn (TSK BSA-ODS) connected via the electrically driven six-port switching column valve from a programmable autosampler to a narrow-bore reversed-phase analytical column (Zorbax Eclipse× DBC_{18}). The present paper describes the development and validation of this robust assay procedure for IND in animal plasma. This procedure has successfully been applied to provide pharmacokinetic data in many diverse pharmaceutical studies, reducing the time taken and improving precision.

2. Experimental

2.1. Chemicals

Indomethacin (pharmacopoeial grade) was obtained from The Society of Japanese Pharmacopoeia (Tokyo, Japan). The IND plaster (26.25 mg/sheet) and cataplasm (22.75 mg/sheet) consisted of an auto-adhesive medicated gauze pad of standard dimensions (7×10 cm) produced by Saitama Daiichi (Saitama, Japan). HPLC reagent grade methanol and water were purchased from Kokusan (Tokyo, Japan). Other reagents used were of analytical grade.

2.2. Animal experimentation

Male Wistar rats weighing 250 g were purchased from Nihon SLC (Tokyo, Japan) and acclimatized over 1 week to our standard environmental conditions (temperature 22–23°C, relative humidity 60–65%, 12 h light–12 h dark). IND plaster (10.5 mg/kg) and cataplasm (9.1 mg/kg) were respectively administered topically to male Wister rats with a 2.5×3 cm drug-impregnated sheet after the hair of the back area of each rat was carefully removed with an electric clipper 24 h prior to application of the formulation. Blood samples were collected from the jugular vein cannula at 2, 4, 6, 8, 10, 12, 16, 24, 28, 36 and 48 h after topical application, and were centrifuged to obtain the plasma. All samples were immediately frozen and kept at −40°C prior to assay.

2.3. Apparatus

The chromatographic system with column-switching consisted of a type 1100 series quaternary pump, a 1100 series thermostatic compartment set at 40°C, a 1100 series on-line degasser, a 1100 series variable-wavelength UV–vis absorbance detector operated at 254 nm, and a 1100 series automatic sampler equipped with two electrically actuated six-port Rheodyne valves, one for sample injection and the other for column switching. This apparatus was all obtained from Hewlett-Packard (Germany). The clean-up/enrichment process took place on a TSK precolumn BSA-ODS (4.6×35 mm, I.D., Tosoh, Japan). Separation occurred on a Zorbax Eclipse× DBC_{18} analytical column (4.6×250 mm, I.D., Agilent Technol., USA). The autosample rack was a specially designed aluminum block which could be held at the desired temperature using a thermostat. Data acquisition and integration of the chromatographic peak was carried out using HPLC system control software running on a compatible computer (Hewlett-Packard CHEMSTATION chromatographic management system, Windows).

2.4. Sample preparation

A stock solution (1000 μg/ml) of IND in methanol was prepared in disposable polystyrene tubes

(Becton Dickinson, USA). Calibration standards were prepared daily in the range 50–10 000 ng/ml (50, 100, 300, 600, 1250, 2500, 5000 and 10 000 ng/ml) from serial dilution of the stock solution by spiking 300-μl aliquots of rat plasma. The plasma samples were added to the filter cup from an Ultrafree-MC centrifugal filter kit with a 0.22-μm microporous filter (Millipore, USA) and were centrifuged (13 000 *g*, 10 min) to move particles or small aggregates from the plasma. After ultrafiltration, a 150-μl volume of plasma was transferred into a silanized glass insert vial placed on the sample rack of the autosampler. The needle was rinsed with HPLC grade water between each step to avoid cross-contamination. Having undergone ultrafiltration pretreatment, the plasma samples were determined by the HPLC system, and the peak area of the IND was plotted against the IND concentration to generate calibration curves. The slope and intercept were obtained by least-square linear regression.

2.5. Chromatographic system

The column switching system was fully automated, with the autosampler performing all conditioning, washing, loading operations and data collection. The dual-column system was coupled via an electrically driven six-port switching column valve from the programmable autosampler to a reversed-phase analytical column, allowing enrichment of the analyte and deproteinization of the sample plasma following direct injection. A schematic representation of the apparatus is shown in Fig. 1.

In mode 1 of the switching valve, the plasma samples were directly injected and eluted onto the precolumn, with a washing mobile phase using 0.1% phosphoric acid at a flow-rate of 1 ml/min to remove the plasma proteins and enrich the IND from the plasma sample. By contrast the analytical column in off-line analysis was eluted with a separation mobile

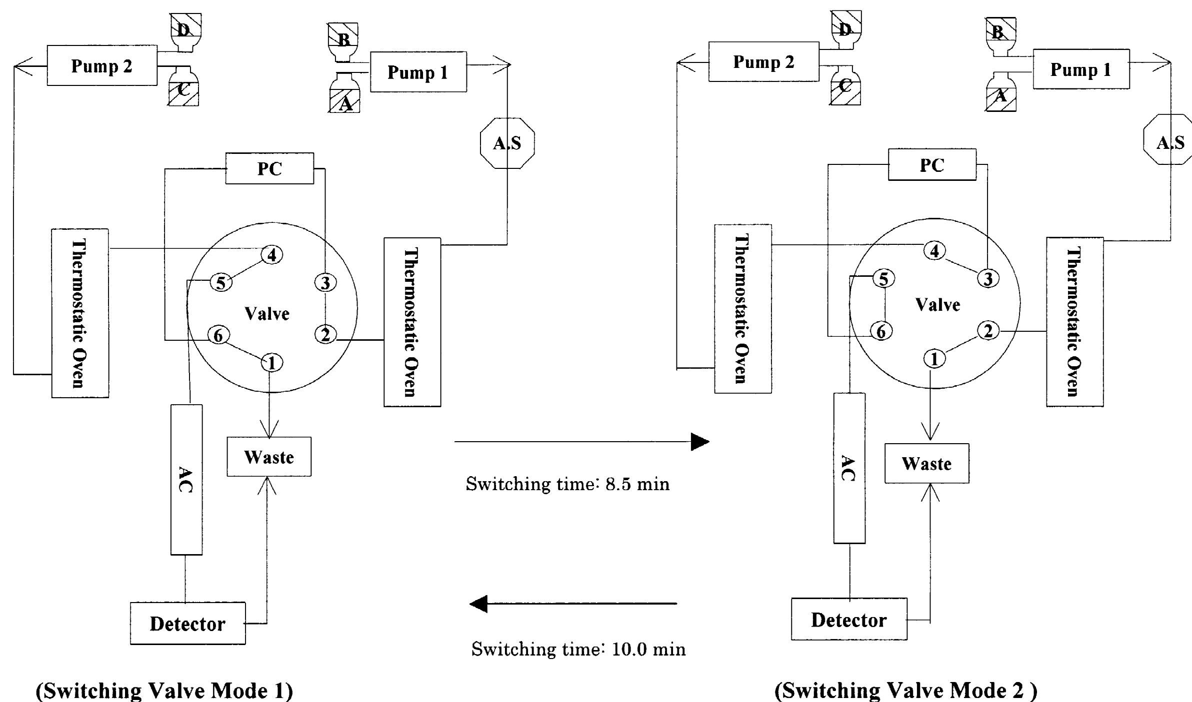

Fig. 1. Schematic diagram of the column-switching HPLC system for on-line separation of indomethacin following direct injection of plasma sample (A,B) mobile phase for pretreatment; (C,D) mobile phase for separation; (AS) autosampler; (PC) TSK precolumn BSA-ODS 08421(4.6×35 mm, I.D.); (AC) Zorbax Eclipse $\times DBC_{18}$ analytical column (4.6×250 mm I.D.).

Table 1
HPLC conditions of the column-switching system

Pretreatment	Column	TSK BSA-ODS 08421 (4.6×35 mm, I.D.)
	Column temperature	40°C
	Mobile phase	0.1% H_3PO_4
	Flow-rate	1 ml/min
Separation	Column	Zorbax Eclipse×DBC_{18} (4.6×250 mm, I.D.)
	Column temperature	40°C
	Detector	UV (254 nm)
	Mobile phase	Methanol–0.1% H_3PO_4 (70:30)
	Flow-rate	1 ml/min

phase of methanol–0.1% phosphoric acid (70:30, v/v) at a flow-rate of 1 ml/min. Eight and a half minutes after injection the switching-valve was turned to mode 2 (backflush mode), coupling the precolumn in line with the analytical column and allowing transfer of the enriched analytes from the precolumn to the analytical column with a separation mobile phase at a flow-rate of 1 ml/min. After backflush for 1.5 min the valve was returned to its original position so that separation was performed with a separation mobile phase at a flow-rate of 1 ml/min; the precolumn was rinsed with a separation mobile phase for 3 min and further reequilibrated with a 0.1% phosphoric acid eluent prior to injection of the next sample. The total analytical time for a single analytical run was approximately 25 min. Quantitation was made by reference to replicate injections of standard IND in blank plasma at a level equivalent to the spiking level. The optimized HPLC conditions for the column switching system are summarized in Tables 1 and 2.

2.6. Pharmacokinetic studies

The model-independent pharmacokinetic parameters following topical administration were determined using a moment analysis program [19]. The area under the plasma concentration–time curve ($AUC_{0-\infty}$), the maximum plasma concentration (C_{max}), the time (t_{max}) at which the plasma concentration peaked, the mean residence time (MRT) and the elimination half-life ($t_{1/2}$), were calculated using the following equations

$$AUC_{0-\infty} = \int_0^t C_p + Ct/\lambda \qquad (1)$$

Table 2
Time program of column-switching HPLC system

Time (min)	Value mode	Pretreatment column	Analytical column	Note
0.0				Injection
0.0–8.5	1	0.1% H_3PO_4	Methanol–0.1% H_3PO_4 (70:30, v/v)	Deproteinization/enrichment
8.5–10.0	2	0.1% H_3PO	Methanol–0.1% H_3PO_4 (70:30, v/v)	Column switching: the transfer of analyte; elution in backflush.
10.0–13.0	1	Methanol–0.1% H_3PO_4 (70:30, v/v)	Methanol–0.1% H_3PO_4 (70:30, v/v)	Column switching: separation/rinse
13.0–25.0	1	0.1% H_3PO	Methanol: 0.1% H_3PO_4 (70:30, v/v)	Separation/analysis; reequilibration
25.0				Stop

$$\mathrm{MRT} = \int_0^\infty tC_\mathrm{p}\,\mathrm{d}t \bigg/ \int_0^\infty C_\mathrm{p}\,\mathrm{d}t \qquad (2)$$

$$t_{1/2} = \ln 2/\lambda \qquad (3)$$

where C_p denotes the plasma concentration of IND at time t after topical application.

3. Results and discussion

3.1. Switching times and elution profile

When developing an on-line HPLC system, the endogenous components should be removed from the plasma by an adequate precolumn in order to prevent precipitation of the plasma proteins and clogging. The packing material used in our precolumn is porous silica gel, with internal and external surfaces of the pores bonded covalently with octadecyl and glycerylpropyl groups, respectively. When the plasma sample is injected the proteins are excluded by their size without adsorption to the external surface, while a small-molecule drug can penetrate into the pore and be retained on the inner surface. This material is therefore suitable in the pretreatment column for on-line HPLC analysis of IND in plasma.

There are two major factors in selecting a suitable mobile for the system: the choice and content of the organic modifier, and the pH of the mobile phase. To prevent the system peaks interfering, miscibility of the mobile phases is also necessary. When an acidic mobile phase (pH 3) was chosen with no organic solvent as a washing eluent for the precolumn, it scarcely denatured proteins and was miscible with the mobile phase used for the subsequent separation. The IND molecule clearly exists in fully unionized ($\mathrm{p}K_\mathrm{a}$ 4.5) form in the mobile phases, so that it is possible to enhance IND recovery and improve the clean-up efficiency in the plasma.

To determine an adequate switching time and optimize chromatographic conditions, a blank plasma sample was injected onto the precolumn, and the elution profile of the sample matrix was measured by direct connection of the precolumn with a UV detector. As shown in Fig. 2, the endogenous components from rat plasma were completely removed from the precolumn within 8.5 min according to a visible analysis of elution profile when the detector signal reached the baseline without any detectable interfering signal; the detector signal corresponding to IND did not emerge for at least 15 min. We therefore chose to make the first valve switching after 8.5 min. Since the total analytical process, including on-line extraction, transfer of analyte, and separation and monitoring, was carried out within 25 min for each sample, the time needed is significantly shorter than with other manual methods.

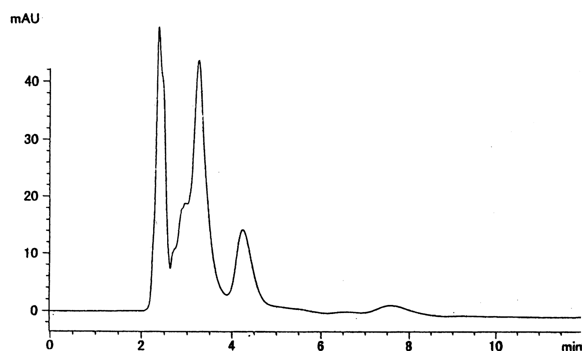

Fig. 2. Elution profile of rat plasma on the precolumn. Blank rat plasma (50 μl) was injected into the precolumn connected directly with UV detector and plasma proteins were eluted out at a flow-rate of 1 ml/min with mobile phase 0.1% phosphoric acid. For other analytical procedures and conditions see Section 2.

3.2. Selectivity and recovery

Selectivity against interference from endogenous compounds found in biological fluids is customarily established by analyzing ten independent sources of the same matrix. As shown in Fig. 3, IND is clearly separated from the endogenous plasma extracted with it, the chromatogram being symmetrical and well resolved without any visible endogenous interference. The retention time for IND was 21.6 min and this was reproducible.

The slopes of regression for the spiked aqueous and plasma ultrafiltrated standards were compared to give estimates of the relative recovery. Slopes of the plasma and aqueous calibration curves at concentrations of 50–10 000 ng/ml (injection volume of 50 μl) were respectively, 165.78 and 167.29, giving a relative deviation of just 1%. A mean relative recovery value of 99% for IND was found throughout the range 50–10 000 ng/ml, indicating that the

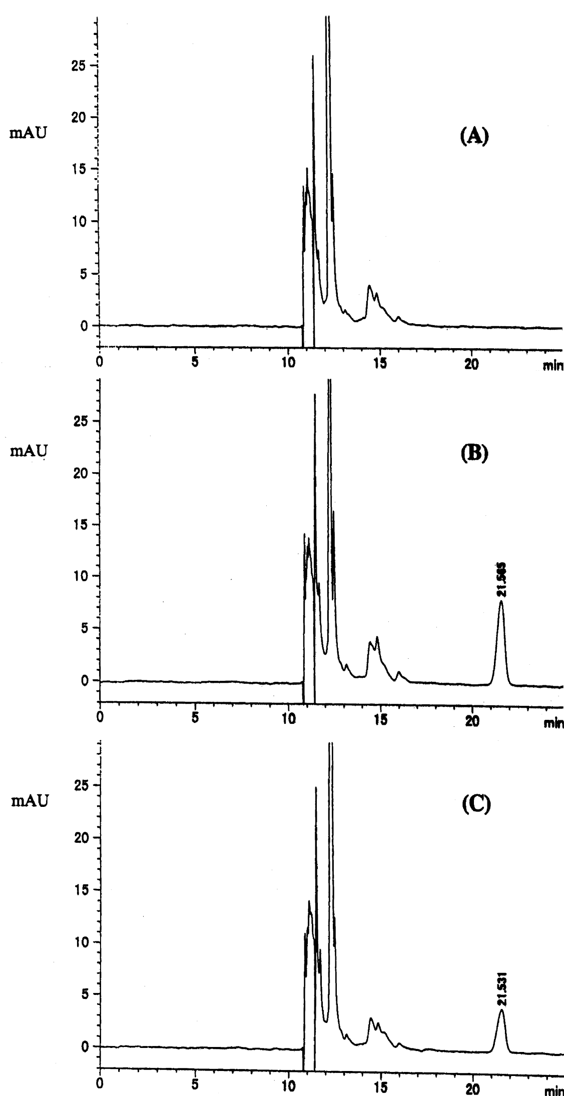

Fig. 3. Representative chromatograms following direct injection of plasma sample into the column-switching HPLC system. (A) Drug-free control plasma; (B) control plasma spiked with indomethacin (2000 ng/ml); (C) plasma sample (764 ng/ml) obtained 2 h after a topical formulation of indomethacin plaster had been applied to the rat skin. For analytical procedure and conditions see Section 2.

precolumn performs excellently at recovering the drug from rat plasma. No internal standard is therefore necessary.

3.3. Linearity, limit of detection and limit of quantification

We studied the relation between the area under the peak and the concentration in plasma samples spiked with known amounts of the drug, for three concentration levels (high, medium and low). The peak-area of the drug was plotted against the drug concentration, and the slope and intercept of the standard curves were estimated by least-square linear regression. The standard curve exhibited excellent linearity (typical equation: $y = 165.78x + 9.723$ in plasma) and correlation coefficient (mean value: $r > 0.999$) in this wide range of concentrations (50–10 000 ng/ml).

The limit of detection was defined as the sample concentration giving a peak area of three times the noise level. Based on the peak baseline noise in ten blank samples, the limit of detection of IND in water was 2 ng/ml. The limit of detection of IND in rat plasma was 10 ng/ml on the basis of the intercept of the regression line and the residual standard deviation [20].

The limit of quantification (LOQ) was defined as the lowest drug concentration which can be determined with a within-day relative standard deviation (RSD) ≤20% and with a value between 95 and 105% of the true value. The LOQ was estimated as 50 ng/ml, so that the present pharmacokinetic analyses are all valid. The mean assay result was 51.41 ng/ml ($n = 10$) with RSD<3%.

3.4. Accuracy and precision

The within-day precision was estimated using the mean and standard deviation (SD) and is expressed as the RSD of the ten replicate results. The between-day precision was estimated by dividing the standard deviation of the within-day means by the average of the within-day means, and is expressed as the RSD. The accuracy of the method expressed as relative mean error (REM) was estimated by calculating the mean percent differences between the mean measured and nominal concentrations from analysis. The acceptance criteria are follows: REM must be within ±15% (±20% at LOQ) for accuracy, and RSD must be within 15% (20% at LOQ) for precision.

Table 3
Within- and between-day precision and accuracy for quantification of indomethacin in plasma

Range	Nominal concentration (ng/ml)	Concentration found (mean±SD) (ng/ml)	RSD[a] (%)	RME[b] (%)
Within-day ($n=10$)				
Low	50	51.41±1.34	2.60	2.82
	300	302.73±7.76	2.56	0.91
Medium	1250	1259.60±14.43	1.15	0.77
	2500	2519.78±18.18	0.72	0.79
High	10 000	9958.64±180.34	1.81	−0.41
Between-day ($n=10$)				
Low	50	51.61±2.47	4.79	3.22
	300	308.98±8.64	2.80	2.99
Medium	1250	1265.41±21.08	1.67	1.23
	2500	2507.78±29.87	1.19	0.31
High	10 000	9938.48±508.78	5.12	−0.62

[a] RSD (%) = 100×(SD/mean).
[b] RME (%) = 100×(mean concentration found − nominal concentration)/nominal concentration.

Table 3 summarizes the precision and accuracy data for indomethacin calculated for the standards in each daily validation run. Accuracy ranged from −0.62 to 3.22% in all levels. The within-day precision as defined above ranged from 0.72 to 2.60%. The between-day precision, expressed as the RSD of the pooled 3-day data, was in the range 1.19–5.12%, showing that the on-line HPLC system gives highly reproducible results.

3.5. Stability

The stock solution of IND was stable for at least 6 months at 4°C. The spiking plasma was frozen and aliquots were thawed and analyzed at specified time intervals. The stability of IND in rat plasma at room temperature was determined by comparing freshly spiked plasma with spiked samples left on the autosampler of the automated HPLC system for 24 h. The mean concentration following this storage period was 98% of the normal values (100, 500 and 2500 ng/ml), demonstrating that the plasma samples were stable. No loss of IND was observed after 3 months of storage at −40°C. No detectable degradation of IND was observed after three freeze (−40°C)–thaw cycles of plasma samples with concentrations of 100, 500 and 2500 ng/ml.

Column stability was also studied for this system. No striking change was observed even after more than 300 plasma samples (50 μl) had been analyzed by this precolumn. However, changing and rinsing of the filter is strongly recommended in order to prolong the lifetime of the column.

3.6. Application of the assay

Our study was designed to study the bioavailability and in vivo pharmacokinetics of indomethacin following topical application to rats. By applying the column-switching technique, at least 50 samples/day can be processed with minimal manual intervention over much of a 48-h period. Centrifuging of the plasma samples made the procedure more reliable by reducing clogging of the system by particles and debris in the plasma. Fig. 4 shows a typical profile of plasma concentration versus time following topical application of IND to the rat. The IND was absorbed and reached its maximum concentration in the

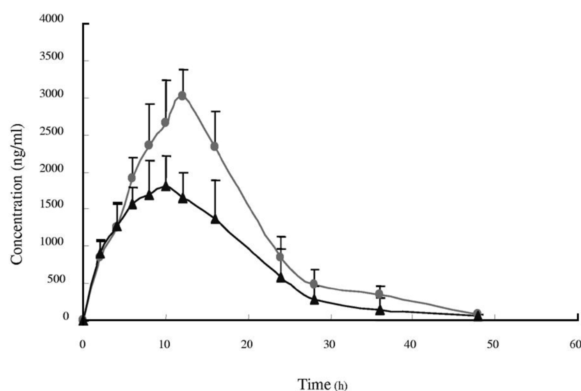

Fig. 4. Plasma concentration–time profile following topical administration of indomethacin in rat. Each symbol with vertical bar represents the mean and standard deviation of at least triplicate samples after topical administration of (●) indomethacin plaster (10.5 mg/kg), (▲) indomethacin cataplasm (9.1 mg/kg) in rats. For analytical procedure and conditions see Section 2.

plasma at around 12 h; the plasma concentration then decreased rapidly up to 24 h and then declined slowly with a half-life of 6.4 h. The MRT and AUC_{0-48} for IND plaster were respectively 15.6 h and 52 821 ng/ml×h. A similar plasma concentration profile for IND cataplasm was observed, with a half-life of 5.4 h, after topical application. The MRT and AUC_{0-48} for IND cataplasm were, respectively 14.1 h and 34 501 ng/ml×h.

4. Conclusion

We have established a rapid, accurate and sensitive automated analytical method for the determination of IND in animal plasma using an on-line column switching HPLC technique with a precolumn packed with biocompatible material. This system has the major advantages of eliminating tedious manual extraction and minimizing manipulation of the biological samples. The precision, accuracy and sensitivity of this assay were all significantly improved. There was no evidence of instability of IND in human plasma following three freeze–thaw cycles or after a long duration of storage (3 months) at −40°C. The run time per sample, from preparation to data acquisition, is considerably reduced compared to manual methods. Furthermore, because the extraction efficiency of the procedure is so high, no internal standard is necessary. The method has been successfully used to provide pharmacokinetic data in a large number of diverse pharmaceutical studies.

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