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SIMULTANEOUS DETERMINATION OF INDOMETHACIN AND ITS METABOLITES IN RABBIT PLASMA BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY

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SUMMARY

A rapid and sensitive high-pressure liquid chromatographic procedure is described for the simultaneous determination of indomethacin and its metabolites, such as N-deschlorobenzoylindomethacin and O-desmethylindomethacin, in rabbit plasma. Acidified plasma (pH = 5.0) was extracted with ethyl ether and *p*-hydroxybenzoic acid was used as an internal standard. The organic extract was reduced to dryness, the resultant residue redissolved in the mobile phase, and aliquots of this solution chromatographed on a Zorbax-ODS column using a mobile phase consisting of an acetonitrile–0.0025% acetic acid (43 : 57 v/v) mixture. The flow-rate was 1.2 ml/min and the effluent was monitored at 254 nm with 0.08 aufs. The sensitivities of this method were 0.02 mg/ml levels of each compound, such as unchanged indomethacin, N-deschlorobenzoyl-indomethacin or O-desmethylindomethacin, in the plasma samples.

INTRODUCTION

Indomethacin is a non-steroid anti-inflammatory, antipyretic and analgesic agent used in the treatment of rheumatoid arthritis. Numerous approaches have been described for the analysis of this substance. Among these, spectrofluorometric (Hucker et al., 1966; Moriyama et al., 1971), thin-layer chromatographic (Strojny and de Silva, 1975), gas–liquid chromatographic (Ferry et al., 1974; Helleberg, 1976; Sibeon et al., 1978), high-pressure chromatographic (Skellern and Salole, 1975) and radio-isotopic (Duggan et al., 1972; Skeith et al., 1968) methods have been applied for the determination of indomethacin in biological fluids. However, fewer methods have been reported for the estimation of O-desmethylindomethacin and N-deschlorobenzoylindomethacin, which represent the two main metabolites of indomethacin.

Although Yesair and Coutinho (1970) separated indomethacin and its two main

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metabolites by anion-exchange chromatography using spectrophotometry for quantitation, this method was shown to be lacking in sensitivity. For present pharmacokinetic work, a simple and fast high-pressure liquid chromatographic method for the simultaneous determination of indomethacin, O-desmethylindomethacin and N-deschlorobenzoylindomethacin from a single plasma extract, is described.

MATERIALS AND METHODS

Materials

Indomethacin, O-desmethylindomethacin and N-deschlorobenzoylindomethacin were gifts from Sumitomo Chemicals (Osaka, Japan). Acetonitrile, acetic acid, ethyl ether, citric acid, sodium phosphate dibasic 12 hydrate and *p*-hydroxybenzoic acid were of guaranteed reagent grade (Nakarai Chemicals, Kyoto, Japan).

Internal standard solution

About 50 mg of *p*-hydroxybenzoic acid was accurately weighed out, transferred to a 50-ml volumetric flask, and dissolved. A 0.125-ml volume of this solution was pipetted to a second 50-ml volumetric flask and diluted with ethyl ether to make up to full volume.

Chromatographic conditions

A Shimadzu LC-2FG high-pressure liquid chromatograph (HPLC) equipped with an SPD-1 UV detector (Shimadzu, Japan) and a 15 cm × 4.64 mm i.d. Zorbax-ODS column (Shimadzu, Japan) was used. The mobile phase consisted of an acetonitrile–0.0025% acetic acid (43 : 57, v/v) mixture. The operating temperature was ambient, and the flow-rate was 1.2 ml/min with an operating pressure of 50 kg/cm^2. The column effluent was monitored continuously at 254 nm with a full-scale deflection of 0.08 aufs, and the chart speed of the recorder was maintained at 2.5 mm/min.

In vivo tests

The solution of indomethacin was prepared by dissolving 40 mg of the bulk drug in 2 ml of distilled water to which 24 mg of sodium bicarbonate had been added.

Rabbits were used to provide in vivo data; they were fasted overnight before dosing. Indomethacin (20 mg/kg) was then administered intravenously into an auricular vein of the rabbit over 15 sec. All blood specimens were collected from the carotid artery by puncture using disposable plastic syringes pre-rinsed with a 1% solution of heparin sodium in normal saline.

Blood samples were withdrawn at 13 appropriate time intervals after drug administration (see Fig. 3 below). The blood samples were centrifuged at 3000 rpm for 5 min to obtain plasma.

Analytical procedures

A 1-ml aliquot of plasma sample (spiked or from dosed rabbits) was pipetted into a 15-ml glass-stoppered centrifuge tube, along with 2 ml of Sørensens citrate buffer (pH = 5.0) and 2 ml of water. The mixture was mixed for 10 sec and extracted with 5 ml of ethyl ether by mechanical shaking for 15 min. After centrifugation for 3 min at 3000 rpm,

3 ml of the ether phase was transferred to another tube to which had been added 1 ml of internal standard at a concentration of 2.5 μg/ml and evaporated to dryness on a water bath at 30°C in vacuo. The residue was redissolved in 200 μl of the mobile phase and 10 μl of this solution was injected into the column for HPLC through a stop-flow injection port.

Calibration curves were prepared, using known concentrations of indomethacin and its two metabolites with plasma, by plotting the concentration of indomethacin or its metabolites (μg/ml of plasma) against the respective peak ratios.

RESULTS AND DISCUSSION

Fig. 1 gives typical chromatograms for *p*-hydroxybenzoic acid, N-deschlorobenzoylindomethacin, O-desmethylindomethacin and indomethacin extracted from both spiked plasma (Fig. 1Y) and dosed plasma samples (Fig. 1Z). Under the chromatographic conditions described above, the retention times of these compounds were 1.5, 2.6, 3.4 and 7.2 min, respectively. As indicated in Fig. 1X, negligible interfering peaks were found in the control plasma specimens. These components were the only peaks seen in both extracts. The complete separation of the components in the chromatograms permitted accurate measurements of small quantities of N-deschlorobenzoylindomethacin or O-des-

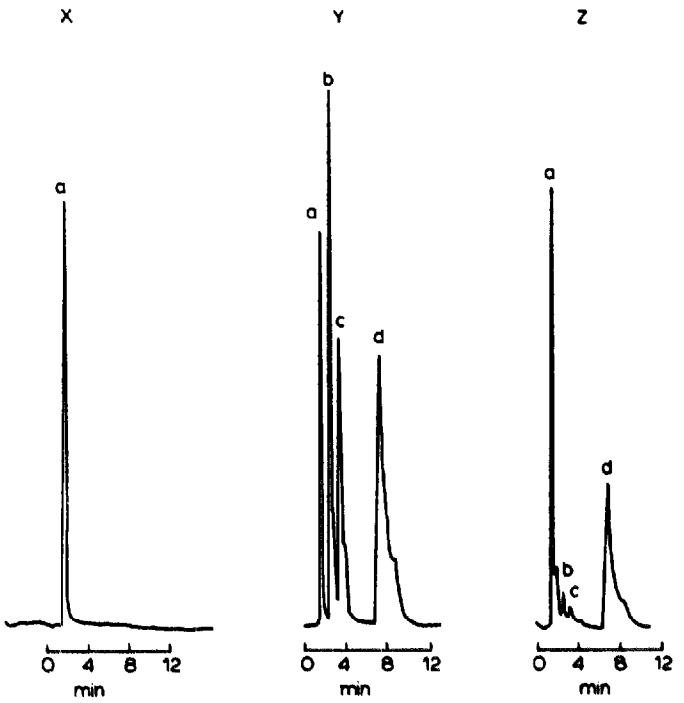


Fig. 1. High-pressure liquid chromatograms of rabbit plasma extracts. Key: X, control plasma containing internal standard (a); Y, plasma containing internal standard (a), N-deschlorobenzoylindomethacin (b), O-desmethylindomethacin (c) and indomethacin (d); Z, plasma sample at 50 min after intravenous administration of 20 mg/kg of indomethacin.

methylindomethacin in the presence of a large quantity of indomethacin.

The mobile phase was chosen as the solvent for redissolving the sample residues since the internal standard, indomethacin and its two metabolites were all soluble in this solvent, the injection of 10 μl of the mobile phase did not affect the separation of the components, and the vapor pressure of the mobile phase was sufficiently high to prevent excessive losses due to evaporation between injections. The *p*-hydroxybenzoic acid was used an internal standard and it was added directly to the extracting solvent. The use of *p*-hydroxybenzoic acid as an internal standard shows particular promise due to its stability and intense UV absorbance at 254 nm.

Fig. 2 illustrates the linearity of the calibration curves for N-deschlorobenzoylindomethacin, O-desmethylindomethacin and indomethacin in plasma at concentrations ranging from 5 to 50 μg/ml. The assay precision and reproducibility are summarized in Table 1. The coefficient of variation (CV) for these results was less than 5% at all concentrations investigated.

The present method employed a solvent of ether for the extraction of indomethacin and its two metabolites from acidified plasma samples. This solvent gave a good recovery of these compounds while yielding essentially no interference from the plasma. The

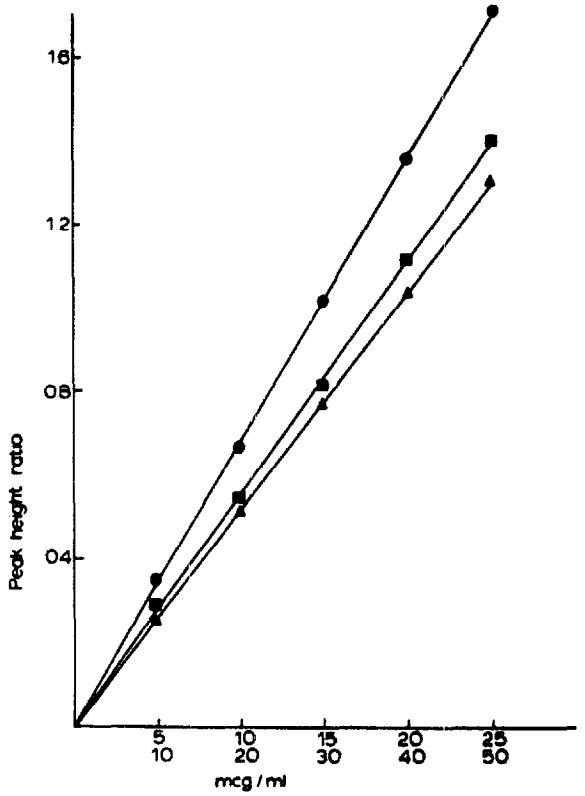


Fig. 2. Calibration curves for indomethacin (■, 10–50 μg/ml), N-deschlorobenzoylindomethacin (●, 10–50 μg/ml) and O-desmethylindomethacin (▲, 5–25 μg/ml) extracted from rabbit plasma. Each point represents the mean of 5 determinations. Linear regression lines: ■, $y = 0.0276x + 0.0083$, $r = 0.9995$; ●, $y = 0.0341x + 0.0007$, $r = 0.999$; ▲, $y = 0.0528x - 0.0058$, $r = 0.9993$.

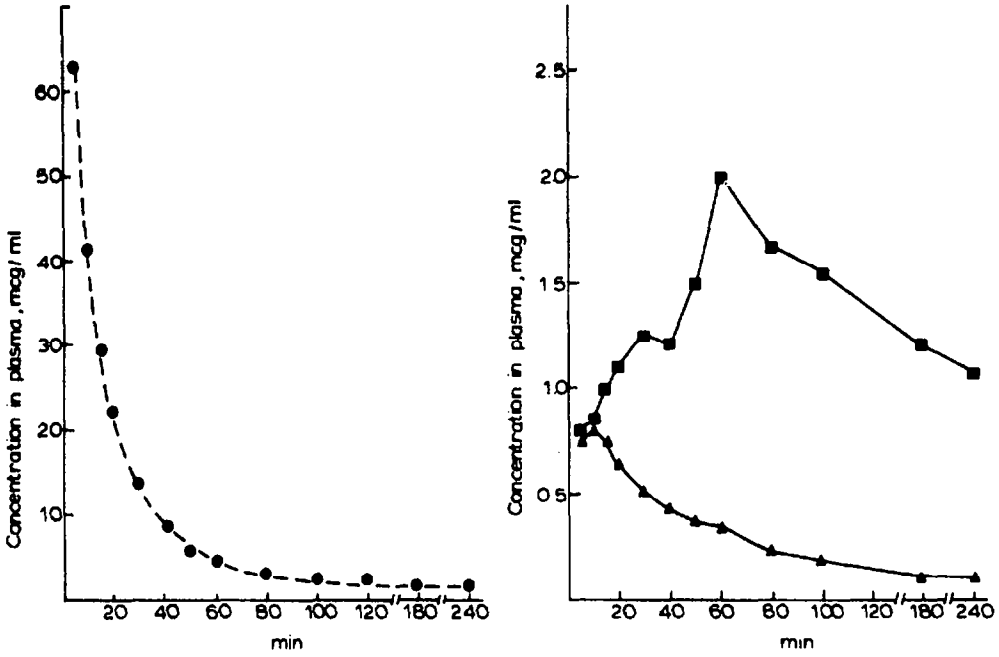


Fig. 3. Plasma concentration–time curves for indomethacin (●), N-deschlorobenzoylindomethacin (■) and O-desmethylindomethacin (▲) in rabbit plasma after intravenous administration of 20 mg/kg of indomethacin. Each point represents the mean of 5 determinations. – – –, curve for indomethacin calculated from the equation; $C = 82.34e^{-4.02t} + 3.83e^{-0.26t}$, where C is indomethacin concentration in plasma.

acidification of the plasma samples enhanced the recovery by solvent extraction due to the acidic nature of these compounds (Helleberg, 1976). Following the procedures described above, the method can accurately and simultaneously measure N-deschlorobenzoylindomethacin, O-desmethylindomethacin and indomethacin in the same sample to concentrations of as low as 0.02 μg/ml.

TABLE 1

ASSAY PRECISION AND REPRODUCIBILITY

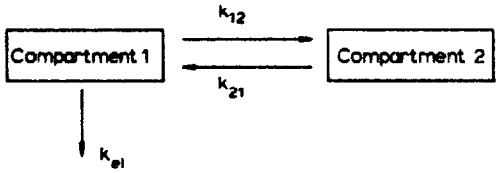
Compound	Concentration in rabbit plasma (μg/ml)	n	Concentration found (μg/ml) Mean ± S.D.	Range	CV(%)
Indomethacin	10	5	10.17 ± 0.34	9.68 – 10.63	3.31
	30	5	29.35 ± 0.48	28.98 – 29.96	1.64
	50	6	50.31 ± 1.45	48.67 – 52.19	2.88
O-Desmethylindomethacin	5	5	5.10 ± 0.12	4.92 – 5.23	2.38
	15	5	14.64 ± 0.23	14.46 – 14.95	1.58
	25	6	25.28 ± 0.75	24.29 – 26.15	2.95
N-Deschlorobenzoylindomethacin	10	5	10.13 ± 0.48	9.61 – 10.73	4.75
	30	5	29.98 ± 0.87	28.60 – 31.00	2.91
	50	6	50.20 ± 2.23	46.65 – 52.58	4.44

TABLE 2

VALUES FOR THE PARAMETERS OF A PHARMACOKINETIC MODEL (CHART 1) DESCRIBING THE METABOLISM AND EXCRETION OF INDOMETHACIN IN RABBITS FOLLOWING INTRAVENOUS ADMINISTRATION OF 20 mg/kg (n = 5)

Parameter	Value
$\alpha(h^{-1})$	4.02
$\beta(h^{-1})$	0.26
$t_{1/2\beta}(h^{-1})$	2.72
$k_{el}(h^{-1})$	2.46
$k_{12}(h^{-1})$	1.40
$k_{21}(h^{-1})$	0.43
V_c(ml)	462
V_T(ml)	1518

α and β are hybrid first-order rate constants and $t_{1/2\beta}$ is the half-life associated with the terminal exponential process; k_{el} = elimination rate constant from the central compartment; k_{12} = rate constant from the central to tissue compartment; k_{21} = rate constant from the tissue to central compartment; V_c = distribution volume of the central compartment; and V_T = distribution volume of the tissue compartment.



Scheme for two-compartment model

Time courses for the concentration of indomethacin and its two metabolites in the plasma of rabbits treated with 20 mg of indomethacin/kg i.v. are given in Fig. 3. The plasma levels of indomethacin appear to be consistent with a two-compartment model. Table 2 summarizes the pharmacokinetic parameters generated from analysis of the data. The N-deschlorobenzoylindomethacin and O-desmethylindomethacin levels are relatively low and their peaks occurred at 1 h and 10 min, respectively.

In conclusion, it can be said that the HPLC procedure described here provides a rapid, sensitive and precise method for the simultaneous determination of plasma levels of indomethacin and its two metabolites. Further applications of the procedure to pharmacokinetic studies of indomethacin will be described later.

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