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Short Communication

Extractionless high-performance liquid chromatographic method for the determination of diclofenac in human plasma and urine

A. Avgerinos, Th. Karidas and S. Malamataris*

Laboratory of Pharmaceutical Technology, University of Thessaloniki, Thessaloniki 54006 (Greece)

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ABSTRACT

An assay using reversed-phase high-performance liquid chromatography with ultraviolet detection, at 278 nm, was developed to measure diclofenac in human plasma and urine at concentrations suitable for biopharmaceutical studies. Indomethacin was used as internal standard and separation was performed at 40°C on a C_{18} Spherisorb column with acetonitrile–0.1 *M* sodium acetate (35:65, v/v) (pH 6.3) as mobile phase. The sample preparation is simple and rapid (extractionless), and the total run time is less than 5 min. The retention time is 2.8 min for diclofenac and 3.6 min for indomethacin. The detection limit is 0.2 μg/ml using a 20-μl loop.

INTRODUCTION

Diclofenac sodium (sodium [*o*-(2,6-dichloroanilino)phenyl]acetate) belongs to the group of the non-steroidal anti-inflammatory agents and appears to combine high therapeutic efficacy and improved tolerability. It is usually given in doses of 25–50 mg, three times daily, or in 100-mg sustained release dosage forms twice per day, and shows rapid absorption followed by almost complete metabolization either by hydroxylation or by glucuro conjugation [1].

Several high-performance liquid chromatographic (HPLC) methods have been developed, during the last twelve years, for the determination of diclofenac alone or together with its metabolites in body fluids (serum/plasma, synovial fluid and urine) [2–15]. One method [2] was developed for screening programmes in systematic toxicological analysis, but two of the other reported HPLC methods lack the sensitivity required for pharmacokinetic investigations [3,4]. The sensitivity of these methods was 1 μg/ml, which is about the peak plasma concentration (C_{max}) after a 100-mg dose of sustained-release tablets or about one quarter the C_{max} arising from a 100-mg oral dose of enteric-coated tablet [10]. Ten other HPLC methods [5–14], although having improved sensitivity, are complex, time-consuming and involve tedious extraction steps. Two of them [7,11] employ electrochemical detection and two additionally involve assays of hydroxylated metabolites [8,13]. The analytical

* Corresponding author.

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characteristics of the published HPLC methods with UV detection are listed in Table I.

Recently an extractionless method has been described with sensitivity down to 0.04 or 0.02 μg/ml (100- or 250-μl loop) in which the serum sample is deproteinized by denaturation with methanol and heating prior to centrifugation and injection [15]. However, this method employs fluorimetric detection. In this communication a similar simple and rapid (extractionless) method with minimum sample preparation is proposed. It is sufficiently sensitive for the determination of diclofenac in both human plasma and urine after oral administration of a single sustained release dose.

EXPERIMENTAL

Apparatus and chromatographic conditions

A Gilson 802G high-performance liquid chromatograph (Gilson, Villiers-le-Bel, France) equipped with a variable-wavelength detector (set at 278 nm) and a Gilson NI chart recorder was used.

Separation was performed at 40°C on a stainless-steel column (25 cm × 4.5 mm I.D.) packed with 5-μm Spherisorb (C_{18} reversed phase, Perkin-Elmer, Norwalk, CT, USA). A stainless-steel precolumn (100 mm × 2.0 mm I.D.) packed with pellicular reversed-phase material (Spherisorb) was used as a guard column. Analytical samples were introduced on to the column using a 20-μl loop valve (Rheodyne, Cotati, CA, USA). The mobile phase was acetonitrile–0.1 *M* sodium acetate (35:65, v/v) with a final pH of 6.3, which was adjusted by the dropwise addition of glacial acetic acid. The flow-rate was 2.8 ml/min and the inlet pressure *ca.* 20.00 MPa (2900 p.s.i.).

Chemicals

Diclofenac sodium and indomethacin were obtained from Heumann Pharma (Nurnberg, Germany) and Geofarma (Milan, Italy), respectively. Solvents for HPLC were purchased from Carlo Erba (Milan, Italy). All solvents and other chemicals were of analytical-reagent grade and were used without further purification.

Stock solutions (1 mg/ml) of diclofenac sodium and indomethacin (internal standard) were prepared in methanol. The diclofenac stock solution was dissolved in drug-free heparinized plasma and in urine to give standard solutions. The internal standard (indomethacin) stock solution was further diluted in methanol to final concentrations of 1 μg/1.5 ml and 1 μg/ml.

Sample preparation

Plasma samples were prepared by the addition of 1.5 ml of diluted internal standard methanolic solution (1 μg/1.5 ml) to 0.5 ml of heparinized plasma. The samples were vortex-mixed for 30 s, centrifuged at 2105 *g* for 10 min, and a 20-μl aliquot of the supernatant was injected onto the HPLC column.

For the determination of diclofenac in urine, samples of 1.0 ml were analysed after the hydrolysis of glucuronides by the addition of 4 *M* hydrochloric acid (100 μl). After vortex-mixing, the acid-treated samples were left to stand for 10 min at room temperature and 1.0 ml of diluted internal standard methanolic solution (1 μg/ ml) was added. No precipitate was formed on the addition of hydrochloric acid and methanolic internal standard solution. After mixing, 20 μl were injected onto the column.

The quantity of diclofenac present in both plasma and urine was estimated by using the peak-height ratio of drug to internal standard and previously prepared calibration graphs. The calibration graphs were prepared by treating a series of drug-free plasma and urine samples spiked with diclofenac in the ranges 0.5–6.0 and 0.5–30.0 μg/ml, respectively.

RESULTS AND DISCUSSION

Typical chromatograms of drug free plasma and plasma from a volunteer, 5 h after the oral ingestion of 100 mg of diclofenac sodium (Voltaren sustained-release tablet) are shown in Fig. 1A. Chromatograms from the analysis of urine samples from the same volunteer are shown in Fig. 1B.

Investigation of the influence of oven temper-

TABLE I

ANALYTICAL CHARACTERISTICS OF REPORTED HPLC METHODS FOR THE DETERMINATION OF DICLOFENAC IN BIOLOGICAL FLUIDS

Biological fluid	Extraction agent	Column type	Mobile phase
Plasma and synovial fluid	H_3PO_4–hexane–isopropanol	Supersil RP LC-18, 5μm	Methanol–acetonitrile–0.02 sodium acetate buffer
Plasma and urine	H_3PO_4–hexane–isopropanol	LiChrosorb RP-8, 10 μm	Methanol–phosphate buffer pH 7 (6:4)
Plasma	Benzene	Partisil, 5 μm	Acetonitrile–0.01 *M* H_3PO_4 (7:3), pH 2.9
Serum	Acetonitrile and evaporation	Spherisorb 5 μm, RP-8C	Acetonitrile–water (50:50), pH 3.3
Plasma	H_3PO_4–hexane–isopropanol	Resolve C_{18} 5 μm, spherical	Isopropanol–acetonitrile–pH 7 acetate buffer
Plasma and urine	Methyl *tert.*-butyl ether	Hypersil 5 μm, ODS	Acetonitrile–methanol–tetrahydrofuran–water
Plasma and synovial fluid	Hexane	Spherisorb 5 μm, ODS	Methanol–water (63:37), pH 3.3

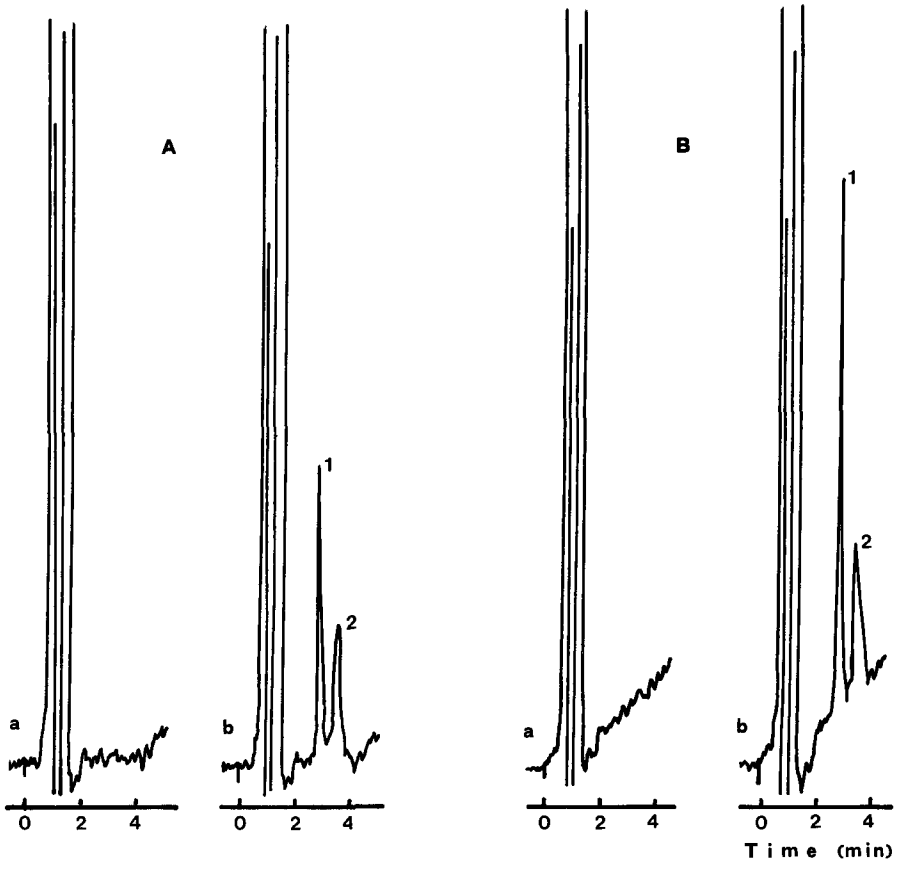

Fig. 1. Chromatograms of (A) plasma and (B) urine samples: a, blank; b, from a volunteer 5 h after oral administration of 100 mg of diclofenac sodium (2.0 μg/ml in plasma and 3.5 μg/ml in urine). Peaks: 1 = diclofenac and 2 = internal standard (indomethacin).

TABLE I (*continued*)

UV detection (nm)	Detection limit (μg/ml)	Analysis time (min)	Internal standard	Reference
215	0.01	7	4 Methoxydiclofenac	5, 6
282	0.01 (plasma) 0.2 (urine)	14	Different metabolites	8
280	0.02	10	Mefenamic acid	9
280	0.025	10	Flufenamic acid	10
210	0.02	21	No I.S.	12
282	0.02	45	No I.S.	13
280	0.01	10	Flurbiprofen	14

ature on the retention behaviour of diclofenac showed an almost proportional shortening of the retention time with temperature increase. An oven temperature of 40°C was therefore chosen because at this temperature viscosity of the solvent mixture was sufficiently low, degassing problems were minimized, less organic solvent was required and the optimum separation power of the system was achieved.

An acid buffer system was used to suppress ionization and to take advantage of the properties of reversed-phase systems. The composition and pH of the mobile phase were varied to achieve the optimum chromatographic conditions. Increasing the percentages of acetonitrile decreased the retention times proportionally and a pH of 6.3 resulted in sharpening of the diclofenac peak. A mobile phase consisting of acetonitrile–0.1 *M* sodium acetate (35:65, v/v) adjusted to pH 6.3 with glacial acetic acid gave optimum resolution of diclofenac and indomethacin (I.S.) and no interference was observed due to endogenous constituents in plasma and urine chromatograms. Particularly in urine, no interfering peaks occurred either with or without the acid treatment. Acid treatment was applied in order to hydrolyse the glucuronide conjugates of unchanged diclofenac and is preferred to alkaline treatment because it avoids possible analytical problems associated with the lability (N-deacylation) of the indomethacin molecules on alkali treatment.

All the calibration graphs of the diclofenac to internal standard peak-height ratios (concentration ranges 0.5–6.0 and 0.5–30.0 μg/ml for plasma and urine, respectively) were linear and almost passed through the origin. The equations of the regression lines were: $x = 0.857y - 0.030$ for plasma and $x = 0.837y - 0.022$ for urine, where x is the concentration of sodium diclofenac (μg/ml) and y is the diclofenac to indomethacin peak-height ratio. The correlation coefficients were 0.999 or better for at least eight points.

TABLE II

REPRODUCIBILITY OF DICLOFENAC SODIUM DETERMINATION IN PLASMA AND URINE SAMPLES ($n = 10$)

Sample	Spiked concentration (μg/ml)	Determined concentration (mean ± S.D.) (μg/ml)	Relative standard deviation (%)
Plasma	1.00	1.00 ± 0.04	4.00
	2.00	2.00 ± 0.07	3.50
Urine	1.00	0.99 ± 0.05	5.00
	2.00	2.01 ± 0.10	4.98

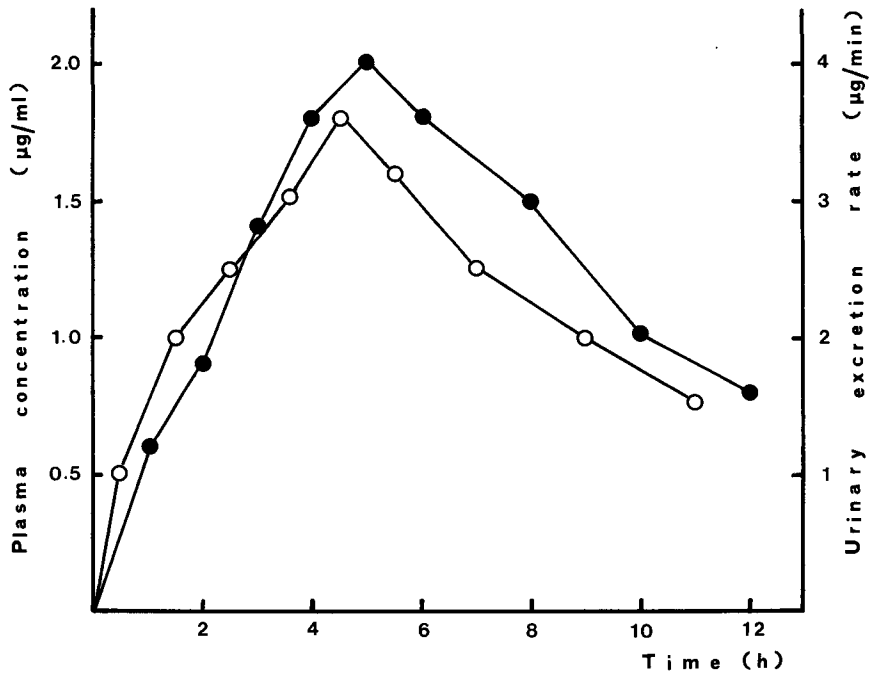

Fig. 2. Plasma concentration (●) of diclofenac and urinary excretion rate (○) *versus* time in a volunteer after the oral administration of et al.

The precision and accuracy of the method were examined for both plasma and urine by repeated analysis of ten samples spiked with diclofenac sodium at concentrations of 1 and 2 μg/ml. The results are given in Table II. Using the criterion of minimum detectability as three times the system noise, the detection limit was 0.2 μg/ml using a 20-μl loop and could be reduced to 0.04 μg/ml by using a 100-μl loop. The relative standard deviations in Table II together with the linearity from 0.2 μg/ml up to 30 μg/ml indicate that the method is suitable for monitoring diclofenac concentrations commonly found in human plasma and urine during therapeutic and biopharmaceutical studies. The method is simple and rapid. About fifty samples can be examined within a working day, since the typical assay time is less than 4 min. For the determination, as little as 0.1 ml of plasma can be used, with a proportional reduction in the volume of the internal standard and the methanol added to the samples.

The method was applied to a hospitalized patient. A single dose of 100 mg of diclofenac sodium (one sustained-release Voltaren tablet) was administered to the patient after an overnight fast. Blood and urine samples were collected at scheduled intervals. Blood samples were collected from a forearm vein and the plasma was separated by centrifugation and was then frozen. Urine samples (5 ml) were frozen immediately after their collection. The plasma concentration–time and the urinary excretion rate–time profiles are illustrated in Fig. 2. The results show that this simple (extractionless) and rapid (about 5 min) method, which utilizes standard HPLC equipment, is sufficiently sensitive to accurately follow blood levels and urinary excretion for at least four half-lives after peak time (T_{max}), as is required in determinations of pharmacokinetic parameters for drug formulation studies.

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