Analysis of Diclofenac and Four of Its Metabolites in Human Urine by HPLC

Ronald J. Sawchuk,[1,3] Jill A. Maloney,[1] Linda L. Cartier,[1] Russell J. Rackley,[2] Keith K. H. Chan,[2] and Henry S. L. Lau[2]

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An HPLC method for the determination of diclofenac (DCF) and four of its metabolites (3′-hydroxydiclofenac, 4′-hydroxydiclofenac, 5-hydroxydiclofenac, and 3′-hydroxy-4′-methoxydiclofenac) in human urine is described. Following base hydrolysis, the samples were neutralized and extracted. Evaporated extracts were reconstituted in mobile phase containing ascorbic acid, and chromatographed, using flow-rate programming, on a reversed-phase column. Absolute recovery (average), was at least 78% for diclofenac and ranged from 75 to 85% for the four metabolites. Standard curves showed linearity over the range of concentrations of 0.2 to 40 ug/mL, using 0.25 mL of urine. Specificity was demonstrated by examining chromatograms of extracts of blank urine from 8 volunteers and 24 study subjects. Good accuracy was observed for all compounds over the concentration range of 0.2 to 40 ug/mL using 0.25 mL of urine. Based on accuracy and precision criteria, the limit of quantitation for all 5 analytes was 0.4 ug/mL, using 0.25 mL of urine. Analysis of urine from subjects with normal and reduced renal function who received diclofenac orally demonstrated that total diclofenac and metabolites excreted in the urine represented approximately 31% and 4% of an oral dose of diclofenac, respectively.

KEY WORDS: diclofenac; diclofenac metabolites; assay; urine; renal failure.

INTRODUCTION

Voltaren (diclofenac sodium) is a nonsteroidal anti-inflammatory drug which has been shown to be effective in the treatment of symptoms of rheumatoid arthritis, osteoarthritis, and ankylosing spondylitis. This drug is extensively metabolized in man to a number of hydroxylated metabolites (Figure 1) which are found in plasma and urine in free and conjugated forms (1,2). These metabolites, as well as unchanged drug, are excreted in the urine and in bile primarily as glucuronide and sulfate conjugates.

Single dose studies in normal subjects who received diclofenac sodium orally showed that mono- and dihydroxy-metabolites were rapidly formed and cleared from the plasma. However, plasma levels of the 3′-hydroxy-4′-methoxydiclofenac metabolite rose gradually, peaking approximately 24 hr after dosing. This metabolite also demonstrated a half-life of about 80 hr, whereas that of the mono- and dihydroxy-metabolites were 1 to 3 hr. In a study in 6 hospitalized patients with chronic polyarthritis (3), these short half-lived metabolites showed no accumulation during multiple dosing, whereas the plasma levels of 3′-hydroxy-4′-methoxydiclofenac increased measurably during continued dosing.

Little information exists concerning the disposition of diclofenac and its metabolites in patients. This results in part from the unavailability of a reliable method for the routine analysis of diclofenac and its metabolites in biological fluids. In 1981, a publication detailed a capillary gas chromatographic method for the analysis of diclofenac and selected metabolites using electron capture detection (2). Although extremely sensitive, this method required extensive sample clean-up before derivatization.

Other chromatographic methods have also been reported for the measurement of diclofenac and several of its metabolites in biological fluids (3-5). One of these (4) is a high-pressure liquid chromatographic method which measures the 5-hydroxy metabolite and parent drug, but does not separate 3′-hydroxydiclofenac and 4′-hydroxydiclofenac. Methods utilizing capillary gas chromatography for analysis of these compounds have good sensitivity and specificity, but are tedious and not suited to routine analysis. It has also been reported that 5-hydroxydiclofenac exhibits instability during assay, and this has been ascribed to oxidation (4,5).

It has been reported that sample extracts must be injected as soon as possible after preparation since degradation of 5-hydroxydiclofenac was observed, although no decomposition of this metabolite was observed during storage of urine samples at −20°C for 5 months, and 3 freeze-thaw cycles during this period had no effect on stability of this metabolite (4). During method validation of a gas chromatographic assay for diclofenac and the four metabolites which are the subject of this study, Luders and Hosieni confirmed the stability of these compounds in urine samples taken through 3 freeze-thaw cycles after storage at −20°C for 70 days (5). The present report describes the validation and application of an analytical method for quantifying concentrations of diclofenac and four of its metabolites (3′-hydroxydiclofenac, 4′-hydroxydiclofenac, 5-hydroxydiclofenac, and 3′-hydroxy-4′-methoxydiclofenac) in human urine in the range of 0.40 to 40 ug/mL. This method involves the addition of ascorbic acid to the mobile phase used to reconstitute the samples prior to injection to provide enhanced stability of 5-hydroxydiclofenac during chromatography. An additional minor metabolite of diclofenac, 4′,5-dihydroxydiclofenac, which is more polar than the other metabolites, elutes with the solvent front and is not assayed using this method.

MATERIALS AND METHODS

Chemicals and Reagents

Diclofenac sodium was purchased from Sigma Chemical Co. (St. Louis, MO). The diclofenac metabolites, (3′-hydroxydiclofenac, 4′-hydroxydiclofenac, 5-hydroxydiclofenac, and 3′-hydroxy-4′-methoxydiclofenac) and internal standard (4′-hydroxy-5-chlorodiclofenac) were obtained

[1] Department of Pharmaceutics, College of Pharmacy, University of Minnesota, Minneapolis, Minnesota 55455.
[2] Bioanalytics and Pharmacokinetics, Ciba-Geigy Corporation, Ardsley, New York 10502.
[3] To whom correspondence should be addressed at Department of Pharmaceutics, College of Pharmacy, Health Sciences Unit F, 308 Harvard Street, Minneapolis, Minnesota 55455.

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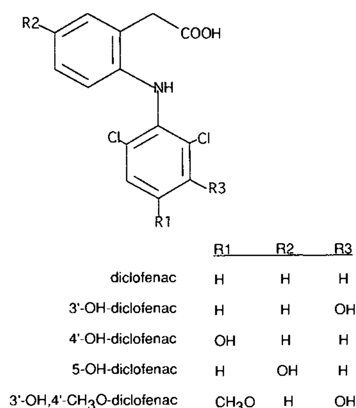

Fig. 1. Structures of diclofenac and four metabolites.

from Ciba-Geigy Corp. (Ardsley, NY). HPLC-grade acetonitrile was obtained from Burdick and Jackson Laboratories, Inc. (Muskegon, MI). HPLC-grade methanol was supplied by E.M. Science (Gibbstown, NJ). Dibasic sodium phosphate and sodium hydroxide were obtained from Fisher Chemical Co. (Fair Lawn, NJ). L-ascorbic acid was purchased from Sigma. Monobasic potassium phosphate was obtained from Mallinckrodt Inc. (Paris, KY). Human urine was obtained from normal volunteers (5 male and 3 female) and was used as matrices from individual subjects, or as a pooled matrix (equal portions) from all subjects.

Preparation of Reagents, Standard Solutions, and Quality Control Samples

1.0 M KH_2PO_4 and 1.0 M Na_2HPO_4 solutions were prepared by weighing 136.09 g KH_2PO_4 and 141.96 g Na_2HPO_4 dissolving each in 1 liter of water. The 1.0 M solutions were sonicated to ensure dissolution. A volume of 877 mL of 1.0 M KH_2PO_4 was mixed with 123 mL of 1.0 M Na_2HPO_4 to prepare a 1.0 M Sorensen's phosphate buffer, pH 6.0.

A 0.1% ascorbic acid in mobile phase solution was prepared fresh daily to prevent the oxidation of 5-hydroxydiclofenac in the reconstituted samples. 20 mg of ascorbic acid was weighed and transferred to a vial containing 20 mL of mobile phase (42.4% $NH_4H_2PO_4$:57.5% MeOH: 0.3% CH_3CN).

A solution of 1.0% ascorbic acid in methanol was prepared. This was diluted 10-fold with methanol to prepare a 0.1% solution.

A 10 mM $NH_4H_2PO_4$ solution was prepared by weighing 1.156 g of $NH_4H_2PO_4$ and dissolving in 1 liter of water. The $NH_4H_2PO_4$ solution is then adjusted to pH 2.66 with approximately 0.3 mL concentrated H_3PO_4. Gradient elution for the chromatography of diclofenac and its metabolites is utilized. The initial composition of the mobile phase is 42.4% 10 mM $NH_4H_2PO_4$, pH 2.66: 57.5% MeOH: 0.3% CH_3CN. The gradient is described below, under chromatography.

A 0.5-mg/mL stock solution of 4'-hydroxy-5-chlorodiclofenac was prepared by weighing 5 mg and diluting to volume in a 10-mL volumetric flask with HPLC-grade methanol. From this stock solution, a working methanolic solution of 12.5 ug/mL was prepared and 50 uL was added to all samples.

Stock solutions (1.0-mg/mL) of diclofenac sodium and its metabolites were prepared separately in HPLC-grade methanol. Working stock solutions for daily standard curves were prepared as follows. A 1.0-mL aliquot from each 1-mg/mL stock solution of diclofenac and the metabolites was transferred into a 10-mL volumetric flask and diluted to volume using 0.1% ascorbic acid in methanol to prepare a 100-ug/mL working stock solution of all the compounds in methanol containing ascorbic acid, 0.15%. Using 0.1% ascorbic acid in methanol as solvent, two serial dilutions (1:10) of this 100-ug/mL stock solution resulted in working solutions containing all compounds at concentrations of 10 ug/mL and 1 ug/mL. All solutions were stored at 4°C when not in use.

Calibration standards containing diclofenac and metabolites were prepared by adding 10- to 100-uL aliquots of each of the respective working stock solutions to 13-mL tapered ground-glass stoppered centrifuge tubes. The methanol was evaporated under nitrogen at ambient temperature. 50 mg of ascorbic acid was added to all tubes. Internal standard solution (50 uL of 12.5 ug/mL 4'-hydroxy-5-chlorodiclofenac) was added to all tubes, except blank. Blank pooled human urine (0.25 mL) was added to each tube to give concentrations of 0.2, 0.4, 1.0, 2.0, 4.0, 10, 20 and 40 ug of each of the analytes per mL of urine. A blank (0 ug/mL) was also included. Calibration standards were prepared on a daily basis.

Quality control samples used during the method validation were prepared as follows. A 2-mL aliquot of each 1-mg/mL diclofenac and metabolite stock solution in 0.1% ascorbic acid solution was transferred using volumetric pipets to a 50-mL volumetric flask. The methanol was dried under vacuum and ambient temperature. Pooled blank human urine was then added to the 50-mL flask to volume. Using pooled blank human urine, serial dilutions of this 40-ug/mL quality control sample were prepared. These quality control samples were separated into 1.0-mL aliquots and stored at −30°C.

Sample Preparation

The standard curve tubes were spiked with DCF and metabolites. After the solvent was evaporated, 50 mg of ascorbic acid was added to the standard curve and quality control tubes. 0.25 mL of pooled blank human urine was added to each standard tube and 0.25 mL of quality control samples were added to appropriate tubes. Internal standard (50 uL of 12.5 ug/mL 4'-hydroxy-5-chlorodiclofenac) was added to all samples, except the blank. From this point, all samples were treated the same. A 0.15-mL aliquot of 5 N NaOH was added to tubes and vortexed at medium speed for 5 - 10 seconds. Samples were capped and hydrolyzed for 1 hr at 70°C. At the end of this time, the samples were cooled to room temperature. The remaining aqueous layer was neutralized with approximately 0.6 mL 1 M HCl and buffered with 0.75 mL of 1M Sorensen's phosphate buffer, pH 6.0. All samples were vortexed and 7 mL of 5% isopropyl alcohol in dichloromethane was added. Samples were shaken horizontally on a mechanical shaker (Eberbach Corp., Ann Arbor, MI) at 180 cycles per min for 10 min and centrifuged at 750g for 5 min. The aqueous layer was aspirated off and approximately 5 mL of the organic layer was transferred to clean tubes. The solvent was evaporated under vacuum at 30°C (Evapo-Mix, Buchler Instruments, Fort Lee, NJ) and sam-

ples were reconstituted with 500 uL of 0.1% ascorbic acid in mobile phase immediately upon removal from the evaporator to minimize any oxidation of 5-hydroxydiclofenac.

Chromatography

Analyses were performed using a Hewlett-Packard Model 1090L HPLC (Hewlett-Packard, Palo Alto, CA) equipped with an automatic injection system, a Shimadzu Model SPD-10A UV spectrophotometric detector, and a Hewlett-Packard 3390A recording integrator set in peak-height mode. The samples (20 uL) were injected onto a reversed-phase (ODS; 5-um, 15-cm, Supelco) column at ambient temperature. Effluent was monitored at 270 nm. The gradient and flow program conditions for the assay are shown in Table I. A total chromatographic time of 30 minutes, including post-run re-equilibration time, was utilized.

Calibration and Sample Quantitation

Daily calibration standards (0.2 to 40 μg/mL) were prepared, extracted, and chromatographed as described above. The chromatographic data were processed as peak-height ratios of analyte to internal standard, followed by weighted (1/nominal concentration) linear least-squares regression analysis of the calibration curve data. Concentrations of diclofenac sodium and its metabolites in the samples were calculated from the corresponding regression equations.

Absolute Recovery

Absolute recoveries (extraction efficiencies) of diclofenac sodium and its metabolites and for the internal standard (4'-hydroxy-5-chlorodiclofenac) were determined by comparing peak-height ratios of the analytes from extracted urine calibration standards to those from unextracted chromatographic standard solutions prepared at equivalent concentrations and chromatographed directly. Indomethacin was utilized as an external standard in the calculation of peak-height ratios.

Metabolite Stability Study

Because of the prolonged analysis time anticipated for a large number of samples, the stability of the metabolites in mobile phase at room temperature was examined over 48 hours. The effect of the addition of ascorbic acid, at a concentration of 0.1%, to mobile phase used to reconstitute samples prior to chromatography on the stability of all metabolites and parent diclofenac was also examined. These studies were conducted in triplicate, and examined the stability of all analytes in amounts of 0.025 and 0.125 ug.

Table I. Chromatographic Flow Rate and Mobile Phase Composition (% by Volume)

Time (min)	Flow (mL/min)	10 mM $NH_4H_2PO_4$	Methanol	Acetonitrile
1.00	1.0	42.2	57.5	0.3
12.00	1.0	42.2	57.5	0.3
14.00	1.6	41.0	57.5	1.5
26.00	1.6	41.0	57.5	1.5
26.01 (postrun)*	1.0	42.2	57.5	0.3

* Postrun period maintained for 4 minutes to allow for re-equilibration.

Linearity, Accuracy and Precision of Calibration Curves

Linearity and inter-day precision of the calibration curves was assessed from a series of 25 curves generated for each of the 5 analytes on different days. Correlation coefficients and the coefficient of variation of the slopes of the regression lines were calculated for the 5 analytes. Accuracy of the method was judged by calculation of the concentration of the calibration standards from these curves. Intra-day precision was assessed by analysis of three calibration curves generated for each of the 5 analytes on the same day.

Accuracy and Precision determined from Quality Control Samples

The overall accuracy and precision of the method was evaluated by the analysis of a set of quality control urine samples spiked with diclofenac sodium and its metabolites of samples on 25 days. Each set consisted of duplicates of three

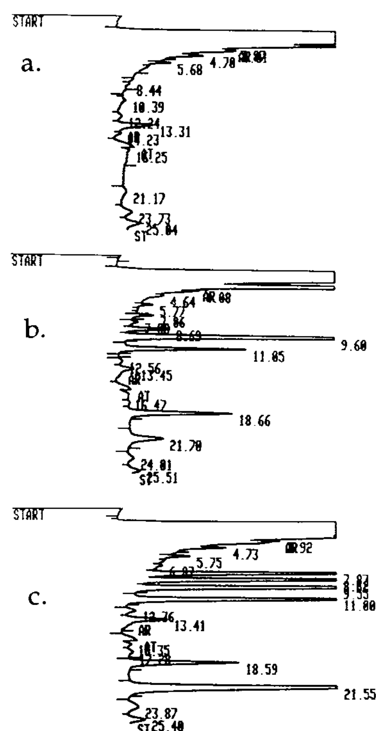

Fig. 2. HPLC chromatograms of extracts of 0.25 mL of urine containing diclofenac and four metabolites. (a) blank pooled human urine. (b) urine from a study subject collected from 4 to 8 hr after an oral dose of 150 mg of diclofenac sodium. Peak 1 (t_r = 7.88 min) is 3'-hydroxy-4'-methoxydiclofenac (BQL); Peak 2 (t_r = 8.69 min) is 3'-hydroxydiclofenac (1.0 ug/mL); Peak 3 (t_r = 9.60 min) is 4'-hydroxydiclofenac (12.0 ug/mL); Peak 4 (t_r = 11.05 min) is 5-hydroxydiclofenac (3.6 ug/mL); Peak 5 (t_r = 21.7 min) is diclofenac (1.2 ug/mL); Peak 6 (t_r = 18.66 min) is 4'-hydroxy-5-chlorodiclofenac (internal standard). (c) quality control sample containing all 5 analytes at a concentration of 10 ug/mL.

Table II. Accuracy and Inter-day Precision (N = 25) in the Analysis of Calibration Curve Samples containing Known Concentrations of Diclofenac Sodium and Metabolites

Nominal concentration (μg/mL)	Diclofenac		5-hydroxy-diclofenac		4′-hydroxy-diclofenac		3′-hydroxy-diclofenac		3′-hydroxy-4′-methoxy-diclofenac	
	Mean	% C.V.	Mean	% C.V.	Mean	% C.V.	Mean	% C.V.	Mean	% C.V.
0.2	105	28.4	92	12.6	99	12.4	97	11.4	100	12.4
0.4	107	27.1	100	11.4	101	10.1	103	9.3	103	9.4
1.0	93	13.6	97	5.2	97	3.8	98	4.3	98	4.8
2.0	93	7.9	97	3.9	98	3.9	99	4.2	99	4.0
4.0	94	4.8	98	3.9	99	3.3	100	3.4	100	3.9
10.0	96	5.6	98	5.0	100	5.2	101	5.2	101	5.2
20.0	97	2.7	97	2.6	99	2.6	100	2.7	100	2.8
40.0	99	1.7	97	1.6	99	1.5	99	1.5	100	1.6
slope	0.24	13.0	0.69	3.0	0.85	2.3	0.75	2.4	0.81	3.4
y-int	0.019		0.021		0.028		0.018		0.009	
r-value	0.999		1.000		1.000		1.000		1.000	

concentration levels of the 5 analytes spanning the range of the assay.

Specificity

Urine from each of eight (five male and three female) normal volunteers was used to examine the variability in the pattern of endogenous peaks, and the effect of this on the analysis of diclofenac and its metabolites. Urine samples containing diclofenac and metabolites were prepared. Internal standard solution was also added to each tube in the series. Blank urine (0.25 mL) from each volunteer was added to all tubes to give concentrations of 1.0 ug of the analytes per mL of urine in the first series, and blanks (0 ug/mL) for each of the urine matrices in the second series. These samples were prepared immediately prior to analysis. The concentrations of diclofenac sodium and metabolites in these samples were determined from a calibration curve constructed using blank pooled urine derived from combining equal volumes of urine from the volunteers.

Specificity of the assay was further assessed in the analysis of blank urines from subjects who participated in the study described below.

Urinary Excretion Rates of Diclofenac and Metabolites in Humans

Urine samples collected from 24 subjects who received diclofenac sodium were analyzed for diclofenac and four metabolites using the method described. This was a single-center, single-dose, parallel-group, open-label trial. Four groups of subjects, stratified by inulin clearance as a measure of renal function, were studied. Diclofenac sodium, 150 mg, was administered orally in buffered aqueous solution to subjects after an overnight fast. Voided urine was completely collected at frequent intervals over the study period of 312 hr. Urine volumes were recorded, and 10-mL aliquots were stored frozen until analysis.

RESULTS AND DISCUSSION

Chromatography

A typical chromatogram of blank pooled human urine is shown in Figure 2a. A chromatogram of an extract of urine from a study subject is also depicted in Figure 2b. The internal standard (4′-hydroxy-5-chlorodiclofenac) was eluted with a retention time of approximately 18.7 min. Diclofenac, 3′-hydroxy-4′-methoxydiclofenac, 3′-hydroxydiclofenac, 4′-hydroxydiclofenac, and 5-hydroxydiclofenac were eluted with retention times of approximately 21.7, 7.9, 8.7, 9.6, and 11.0 min, respectively. A chromatogram of pooled human urine spiked with diclofenac sodium and its metabolites at concentrations of 10.0 μg/mL is also shown in Figure 2c.

Absolute Recovery

Absolute recovery (average), determined by comparison of detector response of chromatograms of extracts to that of unextracted samples, was found to be 78% or greater for diclofenac and to range from 75 to 85% for the four metabolites, determined at three concentrations (0.4, 2, and 20 ug/mL). Mean (SD) recoveries of 80.1% (2.8), 76.8% (6.0) and 85.0% (2.8) were obtained for the internal standard (4′-hydroxy-5-chlorodiclofenac) at concentrations of 0.4, 2.0, and 20 ug/mL, respectively.

Metabolite Stability Study

It had been reported (2) that 5-hydroxydiclofenac is unstable during chromatography, and that the addition of ascorbic acid serves to enhance the stability of solutions of this metabolite. Nevertheless, it has also been shown that this metabolite is stable during storage of urine samples at −20°C, even during freeze-thaw cycling, for periods of 5 months (4) and 70 days (5). The stability of the metabolites in mobile phase at room temperature during an analytical run was examined at approximately 22 and 49 hours. This was investigated because of the long analysis time anticipated for a large number of samples. The effect of ascorbic acid on the stability was also examined. In mobile phase without added ascorbic acid, the stability of all compounds except for that of 5-hydroxydiclofenac was excellent. Approximately 30% of the initial peak-height ratio (PHR) of this metabolite was lost during the 48-hr period. However, ascorbic acid was found to stabilize the compound, as no loss in PHR was

Table III. Accuracy and Intra-day Precision in the Analysis of Calibration Curve Samples (n = 3) Containing Known Concentrations of Diclofenac Sodium and Metabolites

Nominal concentration (μg/mL)	Diclofenac		5-hydroxy-diclofenac		4'-hydroxy-diclofenac		3'-hydroxy-diclofenac		3'-hydroxy-4'-methoxy-diclofenac	
	Mean	% C.V.	Mean	% C.V.	Mean	% C.V.	Mean	% C.V.	Mean	% C.V.
0.2	135	12.8	100	5.2	105	3.6	101	1.8	99	5.7
0.4	106	3.4	99	2.8	101	1.2	100	0.3	101	2.0
1.0	99	3.0	100	1.2	100	0.6	99	0.7	100	1.8
2.0	94	1.8	99	0.7	99	1.3	98	0.9	99	2.1
4.0	101	0.0	101	4.1	101	3.2	103	0.0	99	5.3
10.0	99	0.4	102	1.0	101	0.6	101	0.5	101	0.9
20.0	101	1.4	100	1.2	100	1.3	101	1.2	100	2.0
40.0	100	0.5	100	0.9	100	0.8	99	0.6	100	1.1
slope	0.28	2.4	0.68	0.9	0.87	1.0	0.77	1.2	0.85	1.4
y-int	−0.003		−0.004		−0.001		0.002		0.008	
r	1.000		1.000		1.000		1.000		1.000	

measurable during this period. Indeed, significant loss was observed in the initial analysis (time 0) in both sets of samples (0.025 and 0.125 ug) not supplemented with the antioxidant. This rapid loss may be due to the presence of traces of oxygen in the solution immediately upon reconstitution. However, samples which were reconstituted in mobile phase containing ascorbic acid were stable over the entire analytical period. No significant loss of any of the metabolites other than 5-hydroxydiclofenac was observed over the 48-hr analysis period, even in mobile phase that did not contain ascorbic acid.

Sensitivity (Limit of Quantification)

Differences in the limit of quantification (LOQ) for the 5 analytes were observed, based on accuracy and precision. In order to adopt a common LOQ for the analytes, a conservative value was selected that would apply to all compounds. The LOQ for this method, defined as the lowest analyte concentration with acceptable accuracy and precision for all 5 compounds, was 0.4 ug/mL.

Linearity, Accuracy and Precision of Calibration Curves

The results of the inter-day study for all 5 compounds are shown in Table II, and those for the intra-day study are summarized in Table III. The average correlation coefficients (r) obtained for all compounds were 0.999 or 1.000 in both studies. The regression slopes exhibited coefficients of variation (CV) ranging from 2.3 to 13.0% for inter-day and from 0.9 to 2.4% for intra-day studies, respectively, indicating good reproducibility. Except for the lowest point on the calibration curves (0.2 ug/mL), back-calculated concentrations for the calibration standards from the respective regression lines showed good accuracy over the entire concentration range. As shown in Tables II and III the mean accuracy ranged from 94 to 107%, and 94 to 106%, respectively, over the 0.4 to 40 ug/mL range for all 5 analytes.

Accuracy and Precision Determined from Quality Control Samples

Mean accuracy, as determined in the analysis of the quality control samples (n = 54) at three concentration levels (1.0, 10, and 30 μg/mL) of diclofenac and metabolites ranged from 95 to 109%. The data showed good precision (range, 3.0 to 13% over all concentrations) for the metabolites and parent drug.

Table IV. Amounts of Diclofenac Sodium and Metabolites Excreted in Urine in Subjects with Normal and Impaired Renal Function

	Percent of oral dose				
	Diclofenac	5-hydroxy-diclofenac	4'-hydroxy-diclofenac	3'-hydroxy-diclofenac	3'-hydroxy-4'-methoxy-diclofenac
			Subjects with normal renal function		
Mean	6.2	6.1	16	2.0	0.009
SD	1.8	1.6	3	0.9	
%CV	29	26	17	43	
N	5	5	5	5	1
			Subjects with impaired renal function		
Mean	0.59	0.86	2.4	0.034	0.011
SD	0.47	0.77	1.7	0.059	
%CV	80	89	71	171	
N	5	5	5	3	1

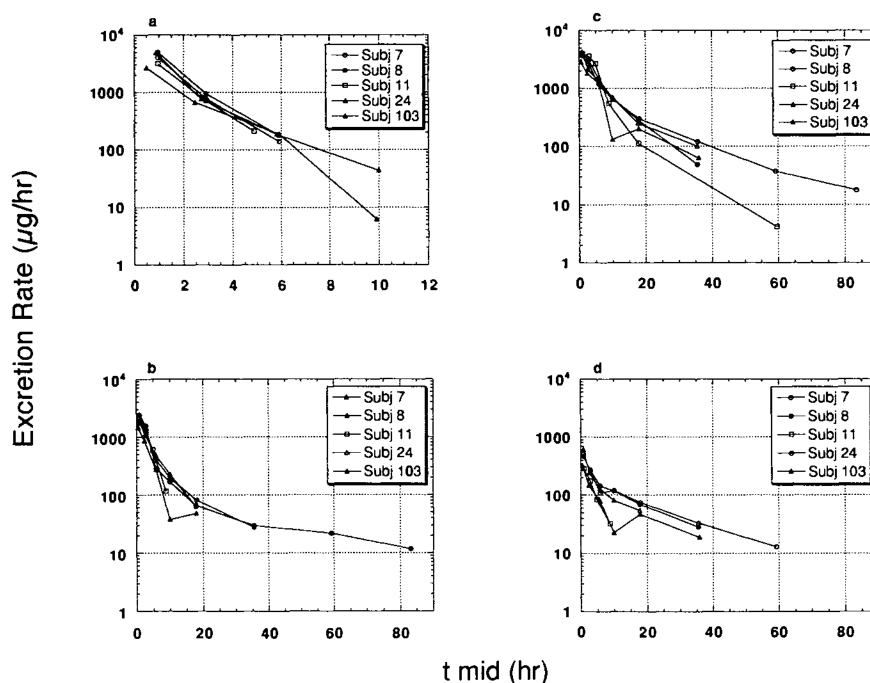

Fig. 3. Excretion rate-time profiles for: (a) diclofenac; (b) 5-hydroxydiclofenac; (c) 4′-hydroxydiclofenac, and; (d) 3′-hydroxydiclofenac in 5 subjects with normal renal function following an oral dose of 150 mg of diclofenac sodium.

Specificity

The specificity of the method was demonstrated by the lack of interferences observed at the retention times of the six compounds upon analysis of control urines pooled from adult volunteers (N = 8) receiving no medication. Nevertheless, it was observed that the urine of 2 of 8 subjects did show the presence of an endogenous peak which interfered with the analysis of 4′-hydroxydiclofenac.

The data showed that the mean accuracy for all compounds, with the exception of 4′-hydroxydiclofenac ranged between 93 and 105%, with CV of 3.1 to 7.1%. For 4′-hydroxydiclofenac, the accuracy averaged 129% (range, 95-264%), with a CV of 46.4%. This was a consequence of a large interfering peak in the hydrolyzed urine of 2 of the 8 subjects. In the 6 subjects who showed no interfering peak, the mean accuracy for 4′-hydroxydiclofenac was 99.8% with a CV of 4.0%.

A peak which interfered with the assay of 4′-hydroxydiclofenac was noted in the blank urine of 1 of 24 subjects whose urines were analyzed for diclofenac and metabolites following administration of oral diclofenac. Similarly, an interfering peak occurred in the analysis of 3′-hydroxydiclofenac in another subject and 3′-hydroxy-4′-methoxydiclofenac in a different subject. No attempt was made to quantitate those particular metabolites in these individuals. In all, 3 interferences were noted in 120 possible subject-compound combinations. The presence of interfering peaks was not related to renal function.

Urinary Excretion of Diclofenac and Metabolites in Humans

Total amounts of diclofenac and metabolites excreted in the urine of subjects with normal (n = 5; inulin clearance > 90 mL/min per 1.73 m^2) and impaired renal function (n = 5; inulin clearance < 30 mL/min per 1.73 m^2) are given in Table IV.

Fractions of the orally administered dose excreted in the urine as DCF and 5-hydroxydiclofenac are comparable with those reported when DCF was administered as a suppository (4). Although those investigators were not able to chromatographically separate 3′-hydroxydiclofenac and 4′-hydroxydiclofenac, they reported that the sum of these was approximately 20% of the dose, in agreement with the present findings. Degen et al. (3), using a specific capillary gas chromatographic analysis, studied 3 healthy subjects and found that 6% of an oral dose was excreted as parent drug, with 13% recovered in the urine as 4′-hydroxydiclofenac. The metabolite with the lowest urinary recovery was 3′-hydroxy-4′-methoxydiclofenac. These results are all in good agreement with those reported here in subjects with normal renal function.

Urinary excretion of DCF, 3′-hydroxydiclofenac, 5-hydroxy-diclofenac, and 3′-hydroxy-4′-methoxydiclofenac each accounted for less than 1% of the oral dose in subjects with reduced renal function. Excretion of the remaining metabolite, 4′-hydroxydiclofenac, represented 2.4% of the dose.

Excretion rates of parent diclofenac and three of its metabolites determined in subjects with normal renal function are plotted versus time in Figure 3. Because the amounts of 3′-hydroxy-4′-methoxydiclofenac excreted in the urine in both groups was negligible, these data were not plotted. The excretion rate-time profile for DCF is consistent with the short elimination half-life (1 to 3 hr) reported for this drug (3). However, the multiphasic profiles observed for the urinary excretion rates of the metabolites 5-hydroxydiclofenac, 4′-

hydroxydiclofenac and 3′-hydroxydiclofenac suggest that the terminal half-lives of these compounds are longer than that of the parent drug.

CONCLUSION

An analytical method for the determination of diclofenac (DCF) and four of its metabolites (3′-hydroxydiclofenac, 4′-hydroxydiclofenac, 5-hydroxydiclofenac, and 3′-hydroxy-4′-methoxydiclofenac) in human urine has been validated, at concentrations in hydrolyzed human urine over the range of 0.4 to 40 ug/mL.

Total amounts of diclofenac and metabolites excreted in the urine of subjects with normal and impaired renal function were approximately 31% and 4% of an oral dose of diclofenac, respectively. Urinary excretion rates of the parent drug and the four metabolites reflected their biological half-lives in all subject groups.

The method should be generally suitable for use in human drug disposition studies. Nevertheless, the presence of endogenous peaks which interfered with the analysis of some of these metabolites was occasionally observed in urine samples obtained from normal volunteers, and from subjects with varying degrees of renal function who participated in a study of the disposition of diclofenac and its metabolites.

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