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Simultaneous determination of ketoprofen enantiomers and probenecid in plasma and urine by high-performance liquid chromatography

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ABSTRACT

A rapid, sensitive, stereospecific reversed-phase high-performance liquid chromatographic method was developed for simultaneous quantitation of ketoprofen enantiomers, probenecid and their conjugates in biological fluids. Following addition of the internal standard, indoprofen, the constituents were extracted into isooctane–isopropanol (95:5), water-washed, extracted with chloroform, then evaporated and the residue sequentially derivatized with ethyl chloroformate and L-leucinamide hydrochloride. The formed diastereomers were chromatographed on a reversed-phase column with a mobile phase of 0.06 *M* KH_2PO_4–acetonitrile–triethylamine (65:35:0.1) at a flow-rate of 1 ml/min and a detection wavelength of 275 nm. The minimum quantifiable concentration was 0.5 μg/ml in 100 μl of rat plasma and urine samples. The intra- and inter-day coefficients of variation for this method are $< 10\%$. The assay is successfully applied to a pharmacokinetic study. The simultaneous analysis of probenecid with several other non-steroidal anti-inflammatory drugs was also successful.

INTRODUCTION

Ketoprofen (KT), 2-(3-benzoylphenyl)propionic acid, is a chiral non-steroidal anti-inflammatory drug (NSAID) marketed as the racemate with therapeutic benefit ascribed mainly to the active *S*-enantiomer as are various other 2-aryl-propionic acid (2APA) derivatives [1]. These drugs are known to undergo stereo-selective disposition kinetics [2–4] and for KT this has been shown to be species-dependent [5]. Probenecid (PB), *p*-(dipropylsulfamyl)benzoic acid, is an achiral uricosuric agent with the potential for coadministration with various NSAIDs to obtain simultaneous uricosuric and anti-inflammatory effects [6–9].

A rapid high-performance liquid chromatographic (HPLC) assay for PB [10] is available as are assays for the simultaneous determination of PB and benzyl-penicillin [11], clofibric acid [12] and diflunisal [13] in biological fluids. Additional HPLC assays are reported that require separate sample preparation and alteration of analysis conditions in order to concomitantly measure PB and zidovudine [14], homovanillic acid and 5-hydroxyindoleacetic acid [15], iodopyracet [16]

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and furosemide [17]. Upton *et al.* [18] reported an HPLC method for KT, naproxen and PB that requires the joining of two columns in series and adjustment of mobile phase and flow-rate in order to measure divergent concentrations of KT and PB. Furthermore, the assay is not stereospecific and so does not allow one to investigate the effect of PB on the stereoselective disposition of coadministered KT enantiomers. A limited number of stereospecific HPLC assays have been reported for KT that involve derivatization with ethyl chloroformate and L-leucinamide hydrochloride by Foster and Jamali [19] and Bjorkman [20] or via $SOCl_2$ and *R*-2-phenylethylamine by Sallustio *et al.* [21].

A major metabolic pathway for many 2APA derivatives such as KT as well as PB is the formation of acyl glucuronides [22,23]. As a result, PB may competitively inhibit the formation of KT glucuronides and the renal or biliary excretion of the formed conjugates [7]. Glucuronidation itself may be an enantioselective process, as significant differences have been found *in vitro* for 2-phenylpropionic acid enantiomers [24] and for etodolac enantiomers in the rat [25]. The effect of PB on the formation and clearance of the glucuronides of the enantiomers of two 2APA derivative NSAIDs, carprofen [26] and benoxaprofen [27], has been studied. Unfortunately, the concentration of PB was not quantified in either report.

The simultaneous quantitation of PB in investigating its effect on glucuronidation is imperative in order to ascertain if the interaction is a competitive and dose-dependent process. Initially, we attempted to quantitate PB along with KT using a previously reported stereospecific method for KT [19] but failed due to interference of the PB and internal standard (I.S.) peaks and non-linearity attributable to the extraction solvent volume. In this paper, we describe a modification of this previous method in order to simultaneously assay KT enantiomers, PB and their conjugates in biological fluids.

EXPERIMENTAL

Materials

Racemic ketoprofen was supplied by Rhone Poulenc Pharma (Montreal, Canada). Probenecid and racemic indoprofen, *p*-(1-oxo-2-isoindolinyl)hydratropic acid, were purchased from Sigma (St. Louis., MO, USA). Stock solutions of the above were prepared in 0.01 *M* sodium hydroxide. Ethyl chloroformate was obtained from BDH (Edmonton, Canada) and L-leucinamide hydrochloride from Sigma. All other solutions and reagents employed were of analytical grade.

Other drugs tested

Indoprofen, carprofen, flurbiprofen, piroxicam, clofibric acid (Sigma), fenoprofen (Eli Lilly, Toronto, Canada), cicloprofen (Squibb, Princeton, NJ, USA), pirprofen (Ciba-Geigy, Mississauga, Canada), *R*-ibuprofen (Sepracor, Marlborough, MA, USA), *S*-ibuprofen (Ethyl Chemicals Group, Baton Rouge, LA, USA), etodolac (Ayerst, Montreal, Canada), naproxen (Syntex, Palo Alto, CA,

USA) and tiaprofenic acid (Roussel, Montreal, Canada) were also subjected to this analysis method in order to determine a suitable I.S. for use with KT and PB.

Apparatus and chromatographic conditions

The HPLC system (Waters Scientific, Mississauga, Canada) consisted of a Model M-45 pump, Model 481 UV spectrophotometer, Model 712 WISP automatic sample processor and a Hewlett-Packard (Avondale, PA, USA) Model 3390A integrator–recorder operated at ambient temperature. A 10 cm × 4.6 mm I.D. stainless-steel reversed-phase column packed with octadecyl-bonded silica (5-μm Partisil 5 ODS-3, Phenomenex, Torrance, CA, USA) attached to a 5 cm × 5 mm I.D. guard column packed with 37–53 μm C_{18} material was used. The mobile phase, unless otherwise stated, consisted of 0.06 *M* KH_2PO_4–acetonitrile–triethylamine (65:35:0.1, v/v) pumped at a flow-rate of 1 ml/min. The detector wavelength was 275 nm. The peak-area method (KT enantiomer or PB/I.S.) was used to calculate response.

Sample preparation

To 100-μl rat plasma samples containing KT and PB were added 100 μl of an aqueous solution of 100 μg/ml racemic indoprofen as I.S. To acidify, 100 μl of 0.6 *M* sulfuric acid were added. The sample was extracted with 5 ml of a mixture of isooctane–isopropanol (95:5) after vortex-mixing for 30 s and centrifuging on a Clay-Adams centrifuge for 5 min at 1800 *g*. The organic layer was transfered to clean glass tubes and 5 ml of water were added. Samples were again vortex-mixed (30 s) and centrifuged (3 min). The organic layer was aspirated off, and 200 μl of 0.6 *M* sulfuric acid were added to the remaining aqueous layer. Chloroform (5 ml) was added, and the samples were once again vortex-mixed (30 s) and centrifuged (3 min). The aqueous layer was aspirated off and the organic phase evaporated to dryness (SVC100H Savant Speed Vac concentrator and refrigerated condensation trap, Emerston Instruments, Scarborough, Canada). The residue was reconstituted with 100 μl of 50 m*M* triethylamine in acetonitrile while vortex-mixing for 30 s. A 50-μl volume of 60 m*M* ethyl chloroformate in acetonitrile and 50 μl of a solution of 1 *M* L-leucinamide hydrochloride and 1 *M* triethylamine in methanol were added at 30-s intervals. The reaction was terminated after 2 min by the addition of 50 μl of HPLC-grade water. Aliquots of 10–60 μl of the resulting solutions were injected into the HPLC system.

The conjugated KT in 100–500 μl urine samples was analyzed by the addition of 25–125 μl of 1 *M* sodium hydroxide, respectively, in order to bring about alkaline hydrolysis of the conjugates. The samples were subsequently acidified with 100 μl of 0.6 *M* sulfuric acid in excess of the volume of 1 *M* sodium hydroxide used for hydrolysis. Sample preparation was continued as for plasma. The amount of conjugated drug is taken to be the difference of the hydrolyzed and unhydrolyzed sample.

Standard curves and precision

The KT enantiomers and PB were quantified against standard curves obtained from sets of solutions prepared by spiking 100 μl of blank rat plasma and urine with racemic KT and PB. The spiked samples provided individual KT enantiomer and PB concentrations of 1, 10 and 40 μg/ml when analyzed by this method. Linearity was investigated by the analysis of standardized samples ranging in concentration from 0.5 to 100 μg/ml. Samples containing 1, 10 and 40 μg/ml KT enantiomers in the absence of PB and 1, 10 and 40 μg/ml PB in the absence of KT were analyzed in order to examine the similarity of results obtained in the presence and absence of either drug. The precision of the assay was determined by the analysis of three sets of three standard sample concentrations daily to determine intra-day variability over five separate days to assess inter-day variability. The accuracy was investigated by the comparison of measured to theoretical concentrations.

Extraction yield

The extraction efficiency was evaluated by extracting racemic KT and PB (10 μg/ml) from 100-μl spiked rat plasma and urine samples ($n = 3$) in the absence of I.S. according to the procedure detailed for sample preparation. Exact volumes (4 ml) of each layer were used for each subsequent step in the sample preparation to insure accuracy in determining yield. The residue obtained after evaporation of the chloroformic layer was reconstituted with 200 μl of methanol with vortex-mixing for 30 s, and 100 μl of I.S. were added. Aliquots of 20 μl of the resulting solutions were chromatographed non-stereospecifically using the exact conditions reported for sample analysis with a mobile phase of 0.06 *M* KH_2PO_4–acetonitrile–triethylamine (75:25:0.1, v/v). The eluted peak-area ratios (KT/I.S. and PB/I.S.) were compared with the ratios obtained after direct injection of unextracted samples of equivalent concentrations of the drugs ($n = 6$) serving as reference. Retention times were 3.4, 4.7 and 6.5 min, respectively, for underivatized I.S., KT and PB.

Derivatization yield

To investigate the overall derivatization reaction, 100-μl rat plasma and urine samples ($n = 3$) were spiked with 100 μg/ml racemic KT, PB and I.S. and analyzed in accordance with the reported procedure using the mobile phase described above for extraction yield. The derivatization yield was calculated by comparing the peak areas of the derivatized KT and I.S. diastereomers and PB with the corresponding underivatized racemic KT, I.S. and PB peak areas.

Dosing and sample collection

A 328-g female Sprague–Dawley rat was catheterized by a procedure described elsewhere [5]. Probenecid was prepared by a previously reported method involving dissolution in 1 *M* sodium hydroxide, dilution with physiological saline and

readjustment of pH to 7.4 with 0.1 *M* hydrochloric acid [28]. Probenecid (50 mg/kg) was administered intravenously 30 min prior to the dose of KT. Racemic KT dissolved in polyethylene glycol 400 (10 mg/kg) was then administered intravenously. Blood (0.2 ml) was collected from the jugular vein cannula at 0 (30 min after PB dose), 0.25, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 9.0, 12.0 and 24.0 h after administration of the KT dose. Between each blood sample collection the catheter was heparinized (10 U/ml). The blood was centrifuged and the plasma portion separated. Urine was collected at 0–3, 3–6, 6–9, 9–12 and 12–24 h after KT administration. All samples were frozen immediately at −20°C in containers previously treated with 0.6 *M* sulfuric acid until analyzed to prevent cleavage of the conjugates.

RESULTS AND DISCUSSION

The previously reported stereospecific assay for KT [19] failed to simultaneously quantitate PB due to interference of the fenoprofen (I.S.) peaks with the PB peak, thus necessitating the use of a different I.S. Poor linear correlations were observed utilizing the previously reported extraction solvent volumes of 3 ml [19]. In the previous method, linearity was investigated in the concentration range 0.05–5 μg/ml, and the volume of 3 ml found to be adequate. In attempting to quantify KT and PB simultaneously in the range of 1–40 μg/ml we found an inconsistency characterized by an upturn in the peak-area ratio–concentration curve resulting in higher than expected ratios for concentrations greater than 20 μg/ml. It was found that increasing the extraction volumes to 5 ml resulted in excellent linearity ($r^2 > 0.999$) and consistent peak-area ratios.

Fig. 1 depicts chromatograms of blank rat plasma, plasma spiked with 10 μg/ml of each KT enantiomer and PB and a 6-h plasma sample. The diastereomers of *R*- and *S*-I.S. and *R*- and *S*-KT and PB elute at 6.5, 7.9, 10.7, 12.6 and 19.4 min, respectively. The order of elution of the enantiomers of KT [19] and indoprofen [29] has previously been determined. The 6-h plasma sample contained 0.65 μg/ml *R*-KT, 16.07 μg/ml *S*-KT and 1.39 μg/ml PB. The chromatograms represented in Fig. 2 are of blank rat urine, urine spiked with 5 μg/ml of each KT enantiomer and PB and unhydrolyzed and hydrolyzed urine samples collected from 6–9 h after KT dosing. The unhydrolyzed sample contained 0.99 μg/ml *R*-KT, 2.17 μg/ml *S*-KT and 55.27 μg/ml PB, and when hydrolyzed an identical sample yielded 1.19 μg/ml *R*-KT, 2.91 μg/ml *S*-KT and 59.26 μg/ml PB.

There were no interfering peaks observed. The first eluting peak of I.S. (peak 1, Figs. 1 and 2) was used for quantitation with typical best-fit lines passing through the plasma and urine data points given in Table I. The slopes of the lines describing both KT enantiomers are very similar, thus indicating non-stereoselectivity in the extraction and/or derivatization methods or alteration of either in the presence of PB. The equations describing KT enantiomer or PB in the absence of each other are very similar to those observed when the drugs were analyzed together,

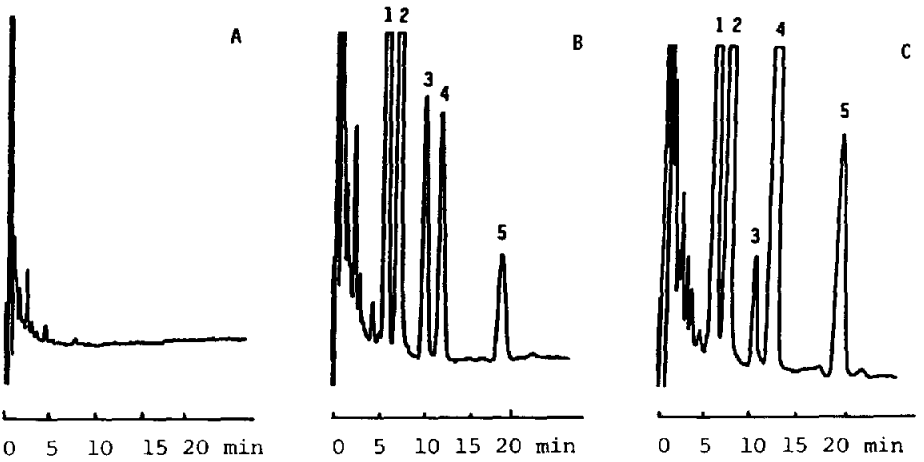

Fig. 1. HPLC profiles of (A) blank plasma, (B) plasma spiked with 10 μg/ml of each enantiomer of KT and PB and (C) a 6-h plasma sample after an intravenous dose of 50 mg/kg PB and 10 mg/kg KT in the rat. Peaks: 1 = *R*-I.S.; 2 = *S*-I.S.; 3 = *R*-KT; 4 = *S*-KT; 5 = PB.

thus, the presence of either drug does not seem to affect the analysis of the other.

The assay is accurate and reproducable as the coefficients of variation (C.V.) obtained for intra-day and inter-day variability over the examined concentration range (1–40 μg/ml) for plasma and urine were less than 10% in each instance.

The detection limit of *R*-KT, *S*-KT and PB was below 0.5 μg/ml, however, reproducable results were obtained only when the concentration was 0.5 μg/ml or

TABLE I

REGRESSION EQUATIONS FOR *R*-KT, *S*-KT AND PB (1–40 μg/ml) TOGETHER AND ALONE IN PLASMA AND URINE

Compound	Regression equation[a]			
	KT and PB together	r^{2b}	KT and PB alone	r^{2b}
Plasma (n = 9)				
R-KT	$y=0.0151x+0.0025$	0.999	$y=0.0163x-0.0069$	0.999
S-KT	$y=0.0154x+0.0033$	0.999	$y=0.0169x-0.0059$	0.999
PB	$y=0.0093x+0.0023$	0.999	$y=0.0089x-0.0007$	0.999
Urine (n = 3)				
R-KT	$y=0.0140x+0.0002$	0.999	$y=0.0259x-0.0088$	0.999
S-KT	$y=0.0143x+0.0001$	0.999	$y=0.0266x-0.0087$	0.999
PB	$y=0.0083x+0.0080$	0.998	$y=0.0126x+0.0148$	0.997

[a] x = drug concentration; y = peak-area ratio of drug/I.S.

[b] r^2 = mean of nine plasma or three urine values.

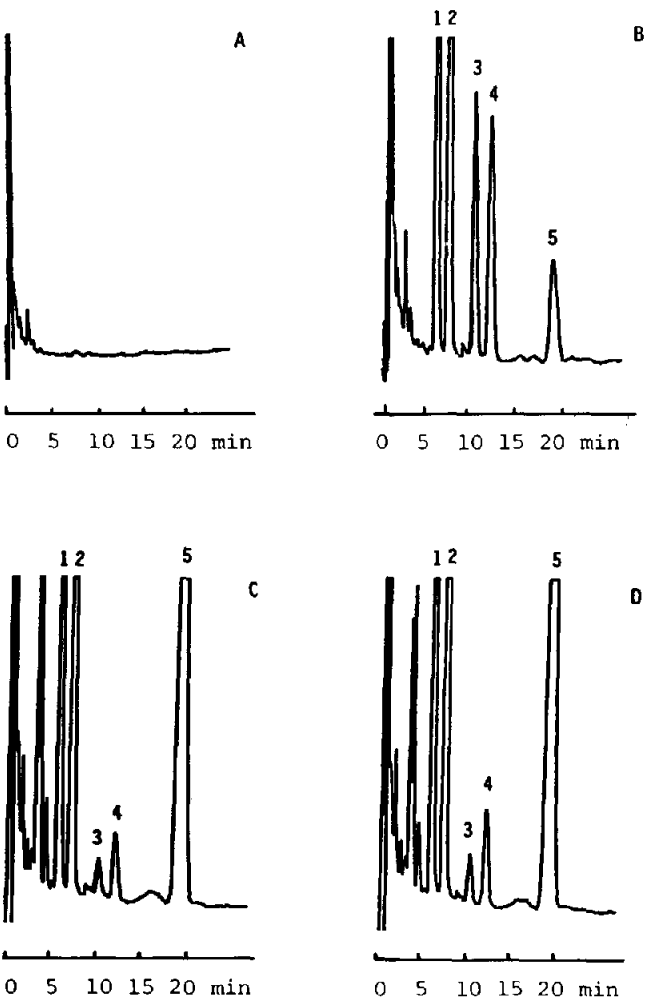

Fig. 2. HPLC profiles of (A) blank urine, (B) urine spiked with 5 μg/ml of each enantiomer of KT and PB, (C) a 6–9 h unhydrolyzed urine sample and (D) a 6–9 h hydrolyzed urine sample after an intravenous dose of 50 mg/kg PB and 10 mg/kg KT in the rat. Peaks: 1 = *R*-I.S.; 2 = *S*-I.S.; 3 = *R*-KT; 4 = *S*-KT; 5 = PB.

higher. Thus, the minimum quantifiable concentration using this assay is 0.5 μg/ml (n = 4, C.V. <9.54%) which was found to be sufficient for determination of concentrations in 100-μl rat plasma samples 12–24 h post-dosing. The plasma concentration–time courses of *R*-KT, *S*-KT and PB in the rat are depicted in Fig. 3 thereby illustrating the application of this assay to the study of their respective pharmacokinetic indices. Sensitivity is expected to proportionally increase with larger sample volumes as would be obtained with human plasma samples.

The extraction yields were 85.81 ± 2.8 and 86.86 ± 7.4% for KT and PB, respectively, from plasma and 92.78 ± 6.2 and 83.88 ± 7.2% for KT and PB,

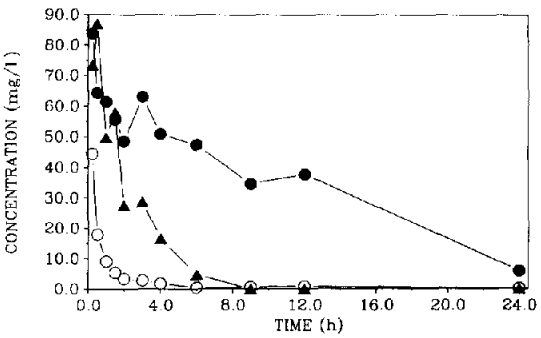

Fig. 3. Plasma concentration–time curve of *S*-KT (●), *R*-KT (○) and PB (▲) following an intravenous dose of 50 mg/kg PB and 10 mg/kg KT in the rat.

respectively, from urine. Derivatization yields obtained were 99.15 ± 0.3, 97.54 ± 0.7 and 84.04 ± 6.2% for I.S., KT and PB, respectively. Accuracy of the analytical method was evaluated, and the results are given in Table II.

In the attempt to find a suitable I.S., flurbiprofen (FL) was initially tried, however, an interfering peak under the *S*-KT peak arising from the FL which became significant only at low concentrations of *S*-KT was apparent. The area of this interfering impurity peak was found to consistently correspond to approximately 1.5% of total *R*- and *S*-FL concentrations in the examined range of 2–10 μg/ml. In addition, injection of FL powder reconstituted in mobile phase into the HPLC system yielded a similar percentage of impurity. Attempts to remove the interfering peak by altering mobile phase, changing detection wavelength, decreasing FL concentration and changing extraction solvents failed. Consequently, numerous compounds as seen under *Other drugs tested* were examined as to

TABLE II

EVALUATION OF ACCURACY

Compound	Theoretical concentration (μg/ml)	Mean calculated concentration (n = 9) (μg/ml)	Coefficient of variation (%)
R-KT	1.0	0.970	9.144
S-KT		0.967	8.319
PB		1.031	8.619
R-KT	10.0	10.477	5.793
S-KT		10.451	6.207
PB		10.470	2.729
R-KT	40.0	40.042	0.376
S-KT		40.091	0.412
PB		40.046	0.748

their suitability as I.S. Although all were derivatized by this method and satisfactory resolution of the enantiomers noted for fenoprofen, cicloprofen and pirprofen, only FL, indoprofen and carprofen diastereomers did not interfere with any of the KT enantiomer or PB peaks. This method was found suitable for the simultaneous determination of indoprofen, carprofen and FL enantiomers with PB in biological samples using KT as I.S. To quantify FL, however, only the *R*-KT peak can be used as I.S. because of the interference of the impurity peak of FL with *S*-KT.

In conclusion, the reported method is rapid, stereospecific and convenient and is applicable to pharmacokinetic studies that require simultaneous determination of PB, KT enantiomers and their conjugates in plasma and urine.

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