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Stereoselective high-performance liquid chromatographic analysis of ketoprofen and its acyl glucuronides in chronic renal insufficiency

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Abstract

A rapid, sensitive method was developed for the quantification of the *R*- and *S*-enantiomers of ketoprofen and their acyl glucuronide conjugates in the plasma and dialysate of hemodialysis-dependent anephric patients. Unconjugated *R*- and *S*-ketoprofen plasma concentrations were determined directly by liquid chromatography using a *S,S*-Whelk-O1 chiral stationary phase. *R*- and *S*-Ketoprofen glucuronide for use as standards were resolved using a C_{18} reversed-phase HPLC column with a mobile phase containing the ion-pair reagent tetrabutylammonium hydrogen sulfate. Plasma glucuronides, however, could not be directly quantified due to matrix interference. Therefore, the glucuronides were isolated using reversed-phase HPLC and quantified after alkaline hydrolysis using the *S,S*-Whelk-O1 chiral stationary phase column.

Keywords: Enantiomer separation; Ketoprofen; Ketoprofen glucuronide

1. Introduction

Ketoprofen [(*RS*)-2-(3′-benzoylphenyl)propionic acid] is a member of the 2-arylpropionic acid (or "profen") class of nonsteroidal antiinflammatory drugs (NSAIDs) and this racemate is widely employed therapeutically (Fig. 1) [1]. In healthy human volunteers *R*-ketoprofen undergoes a small degree of inversion (10–15% of a dose) to the *S*-enantiomer [2]. This constitutes a bioactivation because *S*-ketoprofen is primarily responsible for the characteristic inhibition of cyclooxygenase activity [3,4]. The major pathway of *R*- and *S*-ketoprofen elimination in healthy humans involves the formation of acyl glucuronides (ca. 80% of dose) that are predominantly excreted in the urine (Fig. 1) [1,5].

By virtue of their ability to inhibit prostaglandin synthesis, NSAIDs may induce decrements in renal function (e.g. glomerular filtration rate) in certain risk groups of patients [6]. In particular, patients with pre-existing renal insuf-

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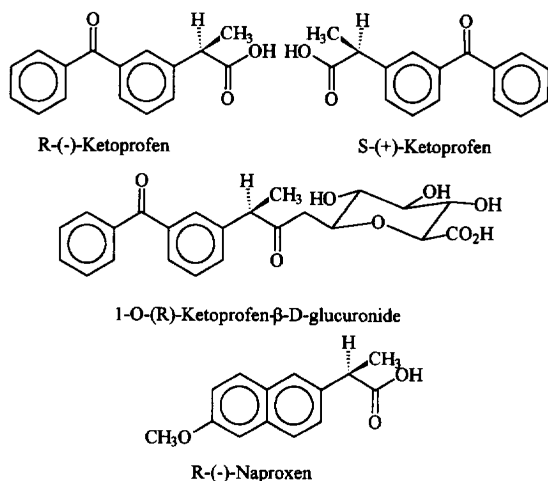

Fig. 1. Structures of the enantiomers of ketoprofen, ketoprofen glucuronide and naproxen.

ficiency are at high risk because renal synthesis of prostaglandins supports renal perfusion in such patients [6]. However, the relative contribution of increased sensitivity to prostaglandin synthesis inhibition and altered pharmacokinetics to the risk in such patient groups is unknown. For drugs, such as ketoprofen, that extensively form acyl glucuronides, significant "futile cycling" can occur wherein hydrolysis of the glucuronide and subsequent reglucuronidation of the aglycone causes these conjugates to accumulate in patients with renal insufficiency [7,8]. Thus concentrations of active drug in plasma increase which may in turn elevate the risk of further reductions in renal function in such patients. We have, therefore, developed robust procedures for quantifying *R*- and *S*-ketoprofen and their acyl glucuronides in the plasma and dialysate of hemodialysis-dependent, functionally anephric patients to assess the magnitude of this potential futile cycling.

Previously reported assays for *R*- and *S*-ketoprofen in plasma have employed tedious and expensive derivatizations with optically active but not optically pure reagents [9–11]. We describe a simple, sensitive, direct assay using a Pirkle-type chiral stationary phase column. Ketoprofen glucuronide concentrations in human plasma and urine have previously been determined by the difference in ketoprofen concentration before and after alkaline hydrolysis [4,12,13]. This indirect method reduces assay precision and limits assay sensitivity, which may be particularly problematic for the quantitation of plasma glucuronides. We describe an alternative method that avoids difference measurements and is robust enough to use in patients with chronic renal insufficiency.

2. Experimental

2.1. Materials

Racemic ketoprofen, was obtained from Wyckoff Chemical (South Haven, MI, USA). *R*-(−)-Ketoprofen, chemical purity (HPLC) 98.6%, enantiomeric excess (ee) 99.00 and *S*-(+)-ketoprofen, chemical purity (HPLC) 99.9%, ee 99.99, were obtained from Sepracor (Marlborough, MA, USA) (Fig. 1). *R*-(−)-Naproxen (*R*-6-methoxy-α-methyl-2-naphthalene-acetic acid) was provided by Syntex (Palo Alto, CA, USA) (Fig. 1). Tetrabutylammonium hydrogen sulfate, ethanol and miscellaneous chemicals (analytical grade) were obtained from Sigma (St. Louis, MO, USA). Acetonitrile was obtained from Mallinckrodt (Paris, KY, USA) and hexane from Baxter (McGraw Park, IL, USA). All solvents were HPLC grade.

2.2. Equipment and chromatographic conditions

Four HPLC systems were used. Systems I and II were used for the analysis and preparative isolation of *R*- and *S*-ketoprofen glucuronides (Fig. 1) in urine respectively. System 1 employed a mobile phase of acetonitrile–potassium phosphate buffer, 50 m*M*, pH 3.0 (30:70, v/v), containing the ion-pair reagent tetrabutylammonium hydrogen sulfate, at a flow-rate of 1 ml/min and UV absorbance detection at 254 nm. Separation of *R*- and *S*-ketoprofen glucuronides was achieved on a 5-μm particle size C_{18} analytical HPLC column (25 cm × 4.6 mm, Ultrasphere, Beckman, San Ramon, CA, USA). System II was used for the preparative isolation of *R*- and *S*-ketoprofen glucuronides from human urine

and was identical to System I except that the column used was a semi-preparative C_{18} 5-μm particle size (25 cm × 10 mm, Ultrasphere, Beckman) and the flow-rate was 3.0 ml/min.

System III used chromatographic conditions that were identical to those in System I except that the mobile phase did not contain tetrabutylammonium hydrogen sulfate. Under these conditions *R*- and *S*-Ketoprofen glucuronide had identical retention times.

System IV was used for the analysis of unconjugated *R*- and *S*-ketoprofen, and *R*- and *S*-ketoprofen after ketoprofen glucuronide hydrolysis. *R*-Naproxen was used as an internal standard. *R*- and *S*-Ketoprofen were separated using a *S,S*-Whelk-01, 5-μm particle size column (25 cm × 4.6 mm, Regis, Morton Grove, IL, USA) and detected using UV absorbance at 254 nm. The mobile phase consisted of hexane–ethanol (90:10, v/v) containing 0.1% acetic acid and was delivered at a flow-rate of 0.9 ml/min.

Fast atom bombardment (FAB) mass spectra of *R*- and *S*-ketoprofen glucuronides isolated from urine were obtained on a Kratos (Ramsey, NJ, USA) MS-50 mass spectrometer set to a resolution of 3000 connected to a D590 data acquisition system. The FAB gun was set at 1 mA and 7 kV. Mass spectrometry was performed in the positive and negative ion modes. The samples were dissolved in glycerol.

2.3. Isolation of glucuronides from human urine

Ketoprofen (75 mg) (Orudis, Wyeth Ayerst Laboratories, Philadelphia, PA, USA) was taken by a healthy volunteer who had not taken any other medications in the preceding two weeks. Urine from each voiding up to 8 h was collected into plastic containers and immediately adjusted to pH 3.0 [14] with glacial acetic acid to stabilize the glucuronide conjugates and stored at −20°C.

To isolate *R*- and *S*-ketoprofen glucuronides from urine, 10-ml aliquots of urine were passed through a preconditioned (1 ml methanol followed by 1 ml 10 m*M* trifluoroacetic acid, pH 3.0) solid-phase extraction cartridge (Sep-Pak C_{18} cartridge, Waters Associates, Milford, MA, USA). The cartridges were then washed with 3 ml 10 m*M* trifluoroacetic acid and the glucuronides eluted with 3 ml acetonitrile–10 m*M* trifluoroacetic acid (80:20, v/v). The eluates were reduced to dryness using a centrifugal evaporator and the residue from each 10-ml aliquot of urine was taken up in 1.5 ml of HPLC System II mobile phase. Two distinct peaks were collected separately from 250-μl aliquots injected onto HPLC System II. The mobile phase was removed by centrifugal evaporation, the residue reconstituted in 250 μl 10 m*M* trifluoroacetic acid and again injected onto HPLC System II. This procedure was repeated one more time and the acetonitrile was removed from the mobile phase by centrifugal evaporation. Buffer salts were then removed using solid-phase extraction as described above. Acetonitrile was then removed from the eluate by centrifugal evaporation and water by lyophilization to give a white crystalline powder which was stored desiccated at −80°C. The final glucuronide standards were free of contamination, based on HPLC and mass spectral analysis and showed no sign of degradation when stored at −80°C for 12 months. The stoichiometric release of ketoprofen from the glucuronide standards following alkaline hydrolysis confirmed their purity.

2.4. Human studies

The human studies were approved by the Institutional Review Board of Indiana University Hospital (Indianapolis, IN, USA). Plasma and dialysate samples were obtained from a patient who gave written informed consent and was undergoing routine hemodialysis three times weekly as a result of end-stage renal failure. Administration of ketoprofen and collection of plasma samples was performed at the General Clinical Research Center (GCRC), Indiana University. The patient was receiving stable doses of chronic medications but did not consume NSAIDs or recreational drugs for the two weeks preceding the study. Pharmacokinetic studies were performed at 07.00 h on a non-dialysis day following single 50-mg dose of racemic ketoprofen (Orudis, Wyeth Ayerst) and following 50-mg every eight hours for eight days. The

regular dialysis schedule was followed. At −0.5, 0.5, 1, 2, 3, 4, 5, 6, 8, 12, 16 and 24 h after ketoprofen administration, blood samples obtained from an intravenous catheter placed in an antecubital vein, were added to heparin containing tubes (Vacutainer, Becton Dickinson, Rutherford, NJ, USA) and immediately centrifuged (3000 *g*, 10 min). Aliquots (1 ml) of plasma and dialysate were placed in plastic vials containing 50 μl 2 *M* sulfuric acid and immediately frozen at −20°C to minimize hydrolysis and intramolecular rearrangement of the acyl glucuronides.

2.5. Sample preparation

To quantify unconjugated *R*- and *S*-ketoprofen, 1-ml aliquots of acidified plasma or dialysate were diluted with 1 ml 10 m*M* trifluoroacetic acid and spiked with 100 μl internal standard solution (0.01 mg/ml *R*-naproxen in methanol). The samples were then acidified to pH 2.0 with 100 μl 2 *M* sulfuric acid and extracted into 2 ml of isooctane–isopropyl alcohol (95:5, v/v). The organic layer was removed, evaporated by centrifugal evaporation and the residue reconstituted in 500 μl of HPLC System IV mobile phase from which 200-μl aliquots were applied to HPLC system IV.

Plasma concentrations of *R*- and *S*-ketoprofen glucuronide were determined by isolating the ketoprofen glucuronide from a 1-ml plasma aliquot. Protein was removed by centrifugation (3000 *g*, 10 min) after precipitation with 2 ml acetonitrile. An aliquot of 2 ml was removed and reduced to dryness with a stream of nitrogen gas. The residue was taken up in 150 μl of HPLC System III mobile phase and 100 μl injected onto HPLC System III. The single peak corresponding to *R*/*S*-ketoprofen glucuronide was collected, 100 μl of internal standard solution added, and the mixture reduced to dryness under a stream of nitrogen. Ketoprofen glucuronide was hydrolyzed using 0.5 ml 0.5 *M* sodium hydroxide (1 h, at room temperature). This sample was then acidified to pH 3.0 with 150 μl 2 *M* sulfuric acid and extracted into isooctane–isopropyl alcohol (95:5, v/v). The organic layer was removed, evaporated to dryness and the residue reconstituted with 200 μl mobile phase from which 100 μl was analyzed using HPLC System IV. The alkaline hydrolysis conditions employed release free 2-arylpropionic acids from primary and intramolecularly rearranged glucuronides [14,15].

2.6. Quantitation of R- and S-ketoprofen in patient plasma and dialysate

For routine analysis of *R*- and *S*-ketoprofen and their glucuronides a 10-point calibration curve was prepared using 1-ml aliquots of bovine serum albumin solution (4%) over a range of 0 to 5 μg/ml for each enantiomer. The calibration curve produced was identical to one using blank plasma. The standards were extracted as described above for unconjugated ketoprofen and a calibration curve constructed for each enantiomer. Quantitation was based on least squares linear regression analysis of concentration versus peak area ratios. The inter-day precision (% coefficient of variation) and accuracy (% relative error) values for the analysis of unconjugated *R*- and *S*-ketoprofen in patient plasma samples were estimated from control ($n=6$) samples made by spiking blank plasma samples with 0.05 or 0.5 μg/ml *R*- or *S*-ketoprofen.

The precision and accuracy of ketoprofen glucuronide quantitation in patient plasma samples was determined by spiking blank patient plasma samples with 0.1 or 1.0 μg/ml *S*-ketoprofen glucuronide. The reference concentration of ketoprofen glucuronide in the stock solution was determined using HPLC System IV subsequent to alkaline hydrolysis. The potential for chiral inversion of *R*- and *S*-ketoprofen glucuronide upon alkaline hydrolysis was determined by hydrolysis (pH 10, 1 h at room temperature) of standard solutions of *R*- or *S*-ketoprofen glucuronide (10 μg/ml). The enantiomeric purity of the glucuronide solution was determined by analysis using HPLC system I using peak-area ratio. After hydrolysis the enantiomeric purity of the resulting unconjugated ketoprofen was determined using HPLC System IV using peak-area ratio.

Dialysate samples were analyzed for both

unconjugated ketoprofen and ketoprofen glucuronide. Unconjugated *R*- and *S*-ketoprofen concentrations in dialysate, to which internal standard solution had been added, were determined by extraction of 10 ml of acidified (pH 2–3 with 2 *M* sulfuric acid) dialysate in to 4 ml isooctane–isopropanol (95:5, v/v) three times. The extracts were pooled and reduced to dryness by centrifugal evaporation, reconstituted in mobile phase and examined using HPLC System IV. *R*- and *S*-Ketoprofen glucuronide in dialysate samples were analyzed either directly or after concentration of 10-ml samples using a solid-phase extraction cartridge as described previously using HPLC System I. Additionally, 10-ml samples of dialysate, to which internal standard had been added, were hydrolyzed (pH 10, 1 h at room temperature) and extracted, after acidification, in to 3×4 ml iosooctane–isopropanol (95:5, v/v). The extract was reduced to dryness by centrifugal evaporation and the residue reconstituted in mobile phase and examined using HPLC System I.

3. Results and discussion

Previously published reports of the enantioselective HPLC separation of many chiral compounds in biological samples, particularly profen NSAIDs such as ketoprofen, have relied upon tedious and expensive precolumn derivatization procedures [9–11]. Often the optically active derivatizing reagents, employed in the latter assays, are not optically pure and therefore the estimated enantiomeric ratios of drug concentrations may be artefactually low. This paper describes a simple, direct procedure for the analysis of *R*- and *S*-ketoprofen and their glucuronides (Fig. 1), following alkaline hydrolysis, which is sufficiently sensitive and robust that it can be applied to the plasma of patients with chronic renal insufficiency. Furthermore, this method avoids the frequently employed approach of quantifying glucuronides using aglycone concentrations before and after hydrolysis [4,12,13], which inevitably reduces assay precision and sensitivity. Because acyl (or ester) glucuronides of xenobiotics, including ketoprofen [8,15] are known to be liable to hydrolysis at plasma pH and in the presence of protein [14], conditions were optimized to stabilize ketoprofen glucuronide in samples, that is samples were immediately acidified and frozen and acidic mobile phases were used. Intramolecular rearrangement of 2-arylpropionic acid glucuronides is often observed and our sample handling conditions would also be expected to minimize the extent this artifact although it has not been explicitly noted to occur for ketoprofen [8,14,15].

The glucuronides of *R*- and *S*-ketoprofen were isolated from human urine for use as standards using HPLC Systems I (Fig. 2) and II respectively. The corresponding retention times were 13.7 and 14.9 min (capacity factor, $k' = 5.5$ and 6.3) for *R*- and *S*-ketoprofen glucuronides respectively in System I and 20.5 and 22.4 min in System II. In the absence of the ion-pair reagent, tetra-

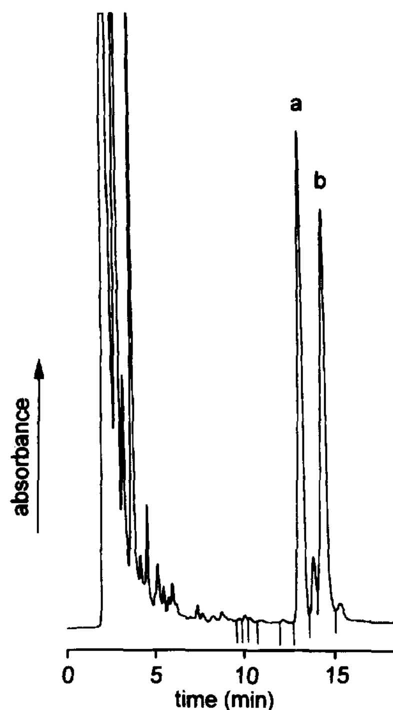

Fig. 2. HPLC chromatographic (System I) separation of *R*- and *S*-ketoprofen acyl glucuronides from the urine of a volunteer given a 75-mg oral dose of ketoprofen. Peaks: (a) *R*-ketoprofen glucuronide ($t_R = 13.7$ min); (b) *S*-ketoprofen glucuronide ($t_R = 14.9$ min).

butylammonium hydrogen sulfate, *R*- and *S*-ketoprofen glucuronides eluted as a single peak (retention times 9.0 and 13.5 min, $k = 4.2$ and 4.0, Systems I and II, respectively). *R*- and *S*-Ketoprofen glucuronides were characterized on the basis of their hydrolytic behavior and by FAB mass spectrometry. The FAB mass spectra of *R*- and *S*-Ketoprofen glucuronide dissolved in glycerol were identical; major characteristic ions at m/z 469.0 and 429.8 corresponding to M + K and M − H respectively were detected in the negative ion spectra. HPLC peaks corresponding to *R*- and *S*-ketoprofen glucuronide disappeared upon alkali hydrolysis with a concomitant increase in the corresponding ketoprofen peak.

Direct analysis of *R*- and *S*-ketoprofen glucuronides in patient plasma samples using HPLC System I was not possible, even after solid-phase extraction of the plasma samples and varying the mobile phase composition and UV detection wavelengths, due to a number of interfering peaks which eluted at similar retention times to *R*- and *S*-ketoprofen glucuronide. These interfering peaks were also present in blank plasma of several hemodialysis patients but not present in the plasma of a healthy subjects. *R*- and *S*-Ketoprofen glucuronides were, therefore, isolated from patient plasma using HPLC System III prior to analysis of unconjugated ketoprofen using HPLC System IV after alkali hydrolysis. The precision and accuracy of this method, determined by adding 0.1 and 1.0 μg of *S*-ketoprofen glucuronide to blank patient plasma samples ($n = 6$) were, coefficient of variation 12.1% and 9.8% and relative error 10.3% and 5.1% for the 0.1 and 1.0 μg/ml samples, respectively. This slight loss of precision and to a greater extent accuracy, compared with the analysis of unconjugated ketoprofen (see below), reflects the additional steps in this procedure. The extent of chiral inversion of *R*- and *S*-ketoprofen glucuronides upon alkali hydrolysis was determined by hydrolysis of a standard solution of *R*- or *S*-ketoprofen glucuronide; the percent inverted was $3.6 \pm 0.3\%$ ($n = 4$) and was independent of the stereochemical configuration of the glucuronide. This error in the determination of the enantiomeric ratio of ketoprofen glucuronides is similar in magnitude to that associated with conventional derivatization assays for unconjugated 2-arylpropionic acids [17]. In procedures which feature both alkaline hydrolysis of acyl glucuronides and derivatization of the free acids, the combination of errors may significantly bias the outcome of pharmacokinetic studies which focus on the enantioselectivity of glucuronide formation.

HPLC chromatograms, using System IV, of unconjugated *R*- and *S*-ketoprofen in a patient plasma sample and *R*- and *S*-ketoprofen derived from alkali hydrolysis of ketoprofen glucuronide isolated from a patient plasma sample are presented in Fig. 3A and B, respectively. The retention times of *R*- and *S*-ketoprofen and *R*-naproxen (internal standard) were 12.7, 13.8 and 17.8 min, respectively ($k' = 4.3$, 5.5 and 7.4). Significantly more *S*- than *R*-ketoprofen was present after hydrolysis of ketoprofen glucuronide in the plasma samples of patients given racemic ketoprofen. The elution of the smaller *R*-ketoprofen peak prior to *S*-ketoprofen allowed for better integration of this peak. However, this elution order may be reversed by using a *R*,*R*-Whelk column as opposed to the *S*,*S* configuration employed in this study.

Estimates of precision and accuracy for the quantification of *R*- and *S*-ketoprofen were obtained from control samples of blank patient plasma spiked with 0.05 or 0.5 μg/ml *R*- or *S*-ketoprofen. The coefficient of variation for *R*-ketoprofen were 9.3% (0.05 μg/ml) and 4.6% (0.5 μg/ml) and for *S*-ketoprofen 6.0% (0.05 μg/ml) and 5.7% (0.5 μg/ml). Relative error values for *R*-ketoprofen were 6.1% (0.05 μg/ml) and 0.09% (0.5 μg/ml) and for *S*-ketoprofen 3.2% (0.05n μg/ml) and 2.4% (0.05 μg/ml). Least squares linear regression analysis of concentrations versus peak-area ratio indicated a high degree of linearity with coefficients of determination in the range 0.997 to 0.999. The limit of detection, defined as the plasma concentration at which the coefficient of determination equals 20%, was approximately 20 ng/ml for each enantiomer.

The plasma concentration–time profile of the *R*- and *S*-enantiomers of ketoprofen and their

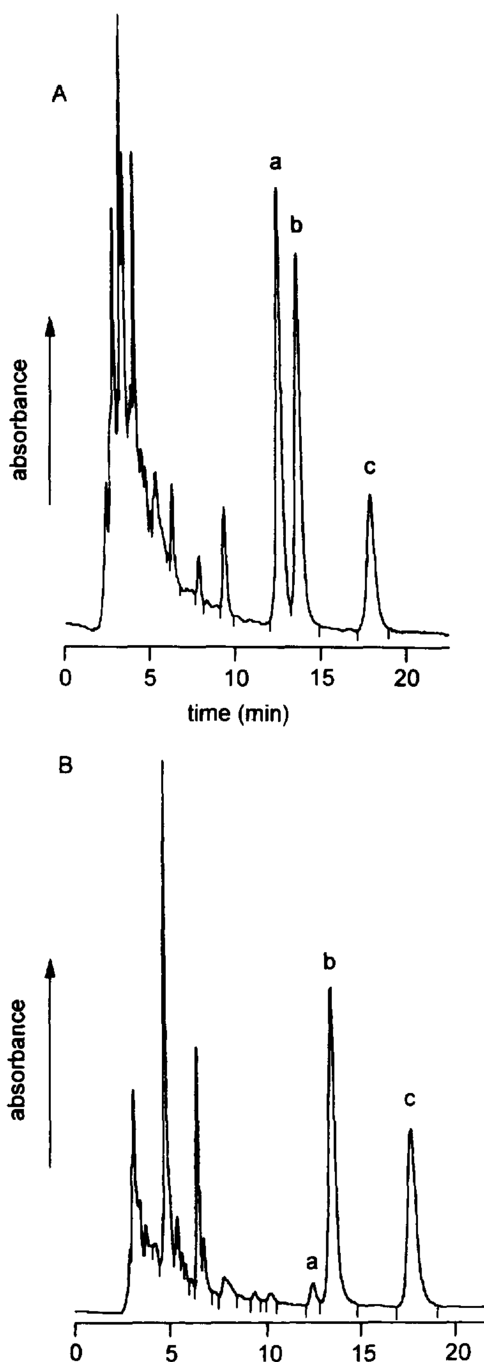

Fig. 3. HPLC chromatogram (System IV) of (A) unconjugated *R*- and *S*-ketoprofen and (B) *R*- and *S*-ketoprofen from the alkali hydrolysis of isolated ketoprofen glucuronide in a plasma sample 2 h after an oral dose of 50 mg ketoprofen. Peaks: a = *R*-ketoprofen (t_R = 12.7 min), b = *S*-ketoprofen (t_R = 13.8 min), c = *R*-naproxen, internal standard (t_R = 17.8 min).

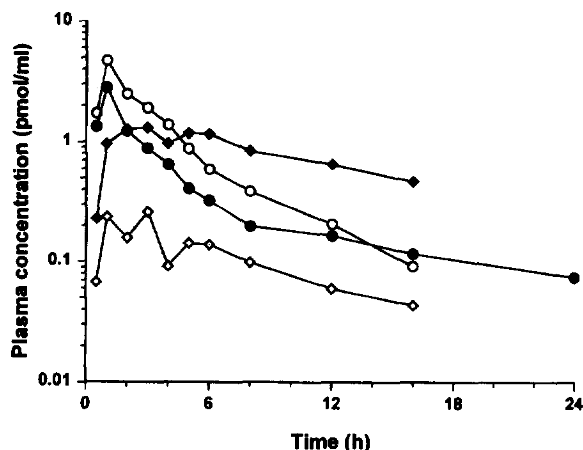

Fig. 4. Plasma concentration versus time profiles of unconjugated *R*-ketoprofen (○), unconjugated *S*-ketoprofen (●), *R*-ketoprofen glucuronide (◇) and *S*-ketoprofen glucuronide (◆) in a functionally anephric hemodialysis-dependent patient given a single oral dose of 50 mg ketoprofen.

acyl glucuronide conjugates in a hemodialysis patient administered 50 mg ketoprofen as a single oral dose is presented in Fig. 4. Both *R*- and *S*-ketoprofen and their glucuronides were detected in patient plasma. The *S*/*R* ratio for ketoprofen area under the plasma concentration versus time curve (AUC) was 0.6 but 9.5 for the corresponding glucuronides. Following eight days of ketoprofen administration (50 mg t.i.d.) to the same hemodialysis patient there was a doubling of the AUC *S*/*R* ratio for ketoprofen to 1.2 and for the corresponding glucuronides to 39.5. This dramatic stereoselectivity in AUCs is substantially greater than that observed in young, healthy volunteers [16], elderly arthritic patients [12] or patients with mild to moderate renal impairment [4]. Only *S*-ketoprofen glucuronide reached a detectable concentration in dialysate (70 ng/ml). Thus dialysis clearance does not appear to be a significant elimination pathway for the enantiomers of ketoprofen or their glucuronides.

In conclusion, we have developed assays for the enantiomers of ketoprofen and their glucuronides in plasma that avoid two pitfalls associated with conventional procedures used in disposition studies of profen NSAIDs. Firstly, our procedure avoids optically impure derivatizing reagents and

secondly, acyl glucuronides are quantified without using difference measurements. The application of these assays to anephric patients receiving hemodialysis indicates that futile cycling and consequent amplification of chiral inversion may occur in such patients.

Acknowledgments

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