ELSEVIER

Journal of Chromatography B, 654 (1994) 61–68

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

High-performance liquid chromatographic enantioselective assay for the measurement of ketoprofen glucuronidation by liver microsomes

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(First received September 27th, 1993; revised manuscript received December 20th, 1993)

Abstract

A stereoselective high-performance liquid chromatographic (HPLC) method was developed to study the *in vitro* glucuronidation of ketoprofen enantiomers by liver microsomes. The HPLC system consisted of a Superspher 100 RP 18 end-capped column eluted with a mixture of acetonitrile and 10 m*M* tetrabutylammonium bromide in 1 m*M* potassium phosphate adjusted to pH 4.3 (30:70, v/v). Ultraviolet detection was performed at a wavelength of 254 nm. The capacity factors of *S*-ketoprofen glucuronide, *R*-ketoprofen glucuronide and *R,S*-ketoprofen were 12.8, 14.5 and 18.1, respectively. Sample pretreatment consisted of protein precipitation in microsomal incubation suspensions and further purification on a Sep Pak C_{18} cartridge before injection onto the HPLC system. Quantitation was performed with standard glucuronides biosynthetized with immobilized microsomes and purified by semi-preparative HPLC. The linearity of the method between 1.25 and 25.0 μg ml^{-1} (coefficient of correlation greater than 0.999), the repeatability (coefficient of variation = 1.2%; $n = 5$), and recovery (within 85%) were tested. The limit of detection was 10 ng for each glucuronide injected. The *in vitro* glucuronidation of *R*- and *S*-ketoprofen was measured in liver microsomes from man and from various animal species (dog, rat, rabbit). For both enantiomers, dog presented the highest specific activity. In contrast, the lowest activity was found in rabbit. On the other hand, the formation ratio of the *S*- and *R*-glucuronides of ketoprofen was close to 1 in man, rat and rabbit, but was 4.5 in dog, thus indicating that the reaction was stereoselective in this species.

1. Introduction

Ketoprofen [2-(3-benzoylphenyl)propionic acid, Profenid®] is a widely used non-steroidal antiinflammatory drug (NSAID) in human. Its structure is chemically related to 2-phenylpropionic acid and has a chiral center. Although administered as a racemate, the *S*-enantiomer in the profen class is generally known to be pharmacologically more active than the *R*-antipode *in vitro* [1].

Ketoprofen is mainly excreted as urinary diastereoisomeric acylglucuronides in man and various animal species [2]. The reaction is supported

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0378-4347/94/$07.00
SSDI 0378-4347(93)E0450-5

by UDP-glucuronosyltransferase (UGT, EC 2.4.1.17) which catalyzes the binding of glucuronic acid from the high energy donor UDP-glucuronic acid onto the carboxyl group of the drug. In contrast to ether glucuronides, which are stable metabolites, acylglucuronides are, at physiological pH, labile and reactive and may undergo undesirable reactions such as isomerization, hydrolysis and adduct formation with serum albumin [3,4]. Indeed, we recently reported that ketoprofen glucuronide could covalently bind to plasma proteins *in vitro* [5]. Glucuronidation has been found to be stereoselective for several chiral compounds depending on the drugs or the animal species considered [6–8]. Therefore the two enantiomers could correspond to two distinct substrate species glucuronidated at different rates or accepted by two different isozymes and thus there is a need to quantitate accurately the formation of the two enantiomer glucuronides.

The optimization of several liquid chromatographic methods used to assay glucuronidation has been performed in our laboratory. Depending on the physicochemical properties of the aglycones, different techniques have been performed using derivatization [9], ion-pairing with hydrophobic counter-ions [10], or column switching [11–13]. Separation of diastereoisomeric glucuronides resulting from conjugation of chiral NSAIDs has been already described [14–17], but such an approach has not been reported for ketoprofen to date.

This work describes the development of a convenient and reliable analytical HPLC method for determining the *in vitro* formation rates of the *R*- and *S*-ketoprofen glucuronides from the racemate, allowing the separation and the quantitation of the two diastereoisomers.

2. Experimental

2.1. Chemicals and reagents

R,S-ketoprofen and pure enantiomeric forms of the drug were a generous gift of Dr. M. Abiteboul (Rhône-Poulenc-Rorer, Paris, France).

UDP-glucuronic acid (sodium salt) was purchased from Boehringer (Mannheim, Germany). Digitonin, D-saccharic acid 1,4-lactone and β-glucuronidase (bovine liver) were obtained from Sigma (St Louis, MO, USA).

All other chemicals and solvents were of analytical reagent grade and were used without further purification.

2.2. Chromatographic conditions

The HPLC system consisted of a two solvent gradient pump (Model Spectra-Physics P 2000, Thermo Separation Products, Les Ulis, France), an injection valve equipped with a 50-μl or a 1-ml sample loop (Model Rheodyne 7125, Cotati, CA, USA), a column oven (Model Croco-Cil, Thermo Separation Products) and a UV-Vis variable wavelength spectrophotometric detector set at 254 nm (Model Spectra-Physics UV 2000, Thermo Separation Products) or a photodiode array detector (Model ABI 1000S, Applied Biosystems, Foster City, CA, USA). All data collection and calculations were performed using an integrator (Model Spectra-Physics Chromjet, Thermo Separation Products).

For the isolation of biosynthetized glucuronides, a semi-preparative column (250 × 10 mm I.D.) prepacked with LiChrosorb (7 μm) RP-18 (Merck-Clevenot, Nogent/Marne, France) was eluted with acetonitrile–water–trifluoroacetic acid (40:60:0.04, v/v/v) at a flow-rate of 2.5 ml min^{-1} and at a temperature of 20°C.

In the case of quantitation of glucuronides during *in vitro* studies, an analytical column (125 × 4 mm I.D.) prepacked with Superspher (4 μm) 100 RP-18 end capped (Merck-Clevenot) was used. The mobile phase used was acetonitrile–10 m*M* tetrabutylammonium bromide in 1 m*M* potassium phosphate adjusted to pH 4.3 (30:70, v/v) and was filtered through a 0.45-μm nylon membrane (SFCC-Shandon, Cergy-Pontoise, France). The flow-rate was 1 ml min^{-1} and the separations were run at a temperature of 35°C.

2.3. Biosynthesis and isolation of glucuronide standards

High amounts of *R*- and *S*-ketoprofen glucuronides were obtained from the corresponding pure enantiomeric drug with rat liver microsomes immobilized into alginate beads as previously reported [18]. Aliquots (1 ml) of the resulting solutions were injected onto the semi-preparative HPLC system. Peak fractions corresponding to the diastereoisomeric glucuronide were collected and the mobile phase was evaporated to dryness with a rotary evaporator at a temperature which did not exceed 40°C. Dried residues were kept at −20°C. Purity estimated by analytical HPLC was over 99.0%.

Stock solutions of biosynthetic glucuronide standards were prepared daily in the mobile phase and kept in the dark at 5°C. Further dilutions were extemporaneously prepared in the same solvent. Calibration curves were calculated using blank microsomal suspensions fortified with glucuronide standards at concentrations 1.25, 2.5, 5.0, 10.0, 15.0 and 25.0 μg ml^{-1}.

2.4. Microsomal sample collection and treatment

A human liver fragment (transplantable liver) was kindly obtained from Pr. E. Singlas and J. Valayer (Hôpital Kremlin-Bicêtre, Paris). Upon removal, the liver sample was kept in liquid nitrogen. The microsomes were prepared according to the method of Dragacci *et al.* [19] and were suspended in 100 m*M* Tris-HCl buffer pH 8.0 containing 1 m*M* EDTA, 1 m*M* dithiothreitol and 20% (v/v) glycerol.

Male Wistar rats (Domaine des Oncins, St Germain l'Abresle, France), weighing 180–200 g, were housed in an environmentally controlled room (12-h light cycle, 22–24°C) and fed a rodent chow (UAR alimentation, Villemoisson, France). Liver microsomes were obtained according to the technique of Hogeboom [20] and stored in 100 m*M* Tris-HCl pH 7.4 containing 0.25 *M* sucrose. Male rabbits (Fauve de Bourgogne, Centre de Production Animale, Olivet, France) were housed under the same conditions, but were fed a rabbit chow (UAR). Their liver microsomes were prepared by the same technique as that used for rats. Liver microsomes from male Beagle dogs were obtained from Iffa-Credo (Domaine des Oncins). The microsomal protein content was determined by the method of Bradford [21], with bovine serum albumin as standard.

The incubation conditions have been previously reported [22]. Briefly, a typical incubation mixture (final volume 200 μl) consisted of microsomal proteins (0.2 mg) previously treated with digitonin at the optimal detergent protein mass ratio of 0.7 for 15 min in an ice-bath, 40 m*M* ketoprofen dissolved in dimethylsulfoxide (5 μl), 100 m*M* D-saccharic acid 1,4-lactone (10 μl), and 100 m*M* acetate buffer pH 5.5 containing 200 m*M* $MgCl_2$ (10 μl). The mixture was preincubated for 1 min at 37°C and the reaction was started by the addition of 20 m*M* UDP-glucuronic acid (30 μl).

After incubation for 20 min at 37°C, the reaction was stopped by the addition of 0.6 *M* glycine–0.4 *M* trichloroacetic buffer pH 2.0–2.2 (200 μl) on ice and the mixture was vortex-mixed. The precipitated proteins were removed by sedimentation in a microcentrifuge for 3 min at 10 500 *g*. The supernatant (300 μl) was passed through a Sep Pak C_{18} cartridge (Millipore, Saint-Quentin-en-Yvelines, France) previously activated with acetonitrile (4 ml) as suggested by Pritchard *et al.* [23]. After washing with 2 ml of water–trifluoroacetic acid (90:10, v/v), the glucuronides were eluted with acetonitrile (2 ml) and the resulting solution was evaporated to dryness at 40°C under nitrogen. The dried residue was redissolved in mobile phase buffer (400 μl) and a 50-μl aliquot was injected onto the analytical HPLC system.

2.5. Identification of ketoprofen glucuronides

Ketoprofen glucuronides were characterized by mass spectrometry and by their susceptibility to hydrolysis by β-glucuronidase. Mass spectrometry ion desorption chemical ionization (DCI, positive mode) was performed on a Nermag R 10-10 spectrometer (Argenteuil, France) in the presence of ammonia. For enzymatic

hydrolysis, assays were run essentially as previously described, except that D-saccharic acid 1,4-lactone, a β-glucuronidase inhibitor, was omitted. Following incubation, 200 μl of β-glucuronidase (bovine liver, 1200 U) dissolved in 0.2 *M* phosphate buffer pH 5.5 were added to the medium. The reaction was carried out for an additional 120 min at 37°C. A control without β-glucuronidase was run simultaneously to estimate the nonenzymatic hydrolysis of the glucuronides. The reaction was stopped by acidification, as previously described, and the products were analyzed by HPLC.

3. Results and discussion

3.1. Development and optimization of the analytical HPLC system

An ion-pairing mode rather than ion suppression in acidic conditions was tested, since the latter is known to give a long elution time of the aglycone. Use of hydrophobic counter ions, such as cethexonium- or tetraheptylammonium bromide did not allow the separation of ketoprofen diastereoisomeric glucuronides. Therefore, a less hydrophobic counter ion such as tetrabutylammonium bromide was used. Among the optimized parameters characterizing the mobile phase (acetonitrile content, counter-ion concentration, pH and ionic strength of the buffer), the pH was the most important factor in obtaining optimum resolution between the two diastereoisomeric glucuronides and the aglycone and optimum selectivity towards the endogenous compounds. The optimum pH found was 4.3 (Fig. 1). It should be noted that the ionic strength of the phosphate buffer could not be increased because this resulted in loss of resolution between the diastereoisomeric glucuronides. The resolution factor between the diastereoisomeric glucuronides was 1.8 and as low as 5% (mole/mole) of each diastereoisomeric glucuronide could be quantified in presence of the other. Under such conditions, the capacity factors of *S*-ketoprofen glucuronide, *R*-ketoprofen glucuronide and *R,S*-ketoprofen

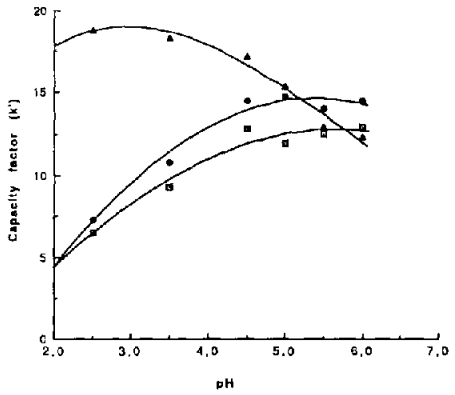


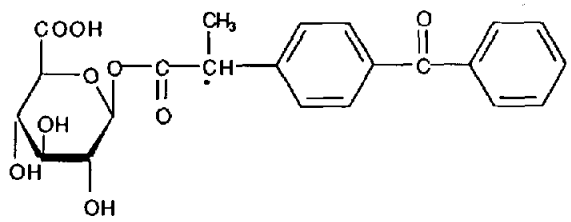
Fig. 1. Variations of capacity factors of *R,S*-ketoprofen (▲), *R*-ketoprofen glucuronide (●) and *S*-ketoprofen glucuronide (▣) as a function of the pH of the buffer of the mobile phase.

were 12.8, 14.5 and 18.1, respectively. Selectivity factors between adjacent peaks were 1.1 and 1.25 and resolution factors 1.8 and 2.0.

3.2. Measurement of the two diastereoisomeric glucuronides in microsomal samples

Quantitation was achieved with standard glucuronides biosynthetized with immobilized microsomes and purified by semi-preparative HPLC [5]. Typically, 4 mg of glucuronide could be obtained from 10 mg ketoprofen after 2 h incubation with the immobilized proteins at 37°C. Analysis by DCI mass spectrometry revealed the structure of the glucuronide (Fig. 2).

The mass fragmentation pattern gave characteristic peaks which corresponded to the molecular ion ($M + NH_4^+$) (*m/z* 448), protonated ketoprofen (*m/z* 255) and ketoprofen $+ NH_4^+$ (*m/z* 272). UV data were established using a diode array detector: no significant spectral shift in maximum wavelength was observed between each glucuronide and its aglycone and thus only one detection wavelength, *i.e.* 254 nm, was used for the three analytes. The purity of fractions containing ketoprofen glucuronides was calculated by measuring the amount of aglycone released by enzymatic hydrolysis. Complete disappearance of the glucuronide peak was observed. The glucuronide/aglycone conversion



Ketoprofen glucuronide (M_r = 430) The asterisk * indicates the chiral center

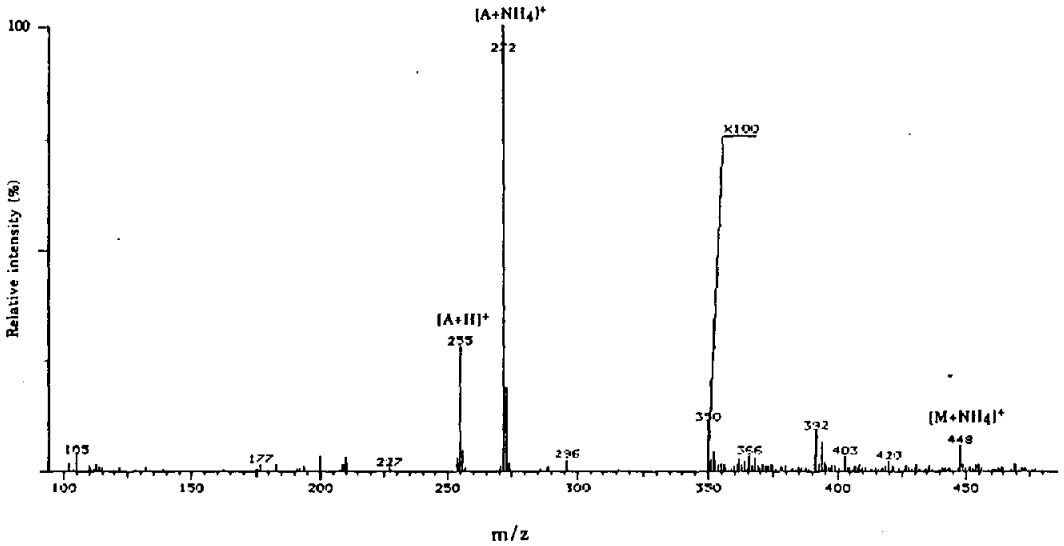


Fig. 2. Desorption in chemical ionization mass spectrometry of *R,S*-ketoprofen glucuronide. The metabolite was produced by rat liver microsomes immobilized into alginate beads, as described in Experimental, and purified by HPLC. The part of the spectrum after 350 (m/z) is expanded 100 times. A: aglycone; M: ketoprofen glucuronide.

ratios calculated from their peak-area ratios were 0.96 and 0.94 for *R*- and *S*-ketoprofen glucuronides respectively.

Since acylglucuronides are known to be unstable at physiological pH [3], the stability of ketoprofen glucuronides during the incubation process and also in solution in the mobile phase was tested. The hydrolysis of the acylglucuronides in the incubation medium was minimized by working at pH 5.5 in the presence of D-saccharic acid 1,4-lactone, a β-glucuronidase inhibitor. In solution in the mobile phase, the peak areas of the glucuronides show no significant decrease over a 2-h period.

The linearity of the method was tested in the biological matrix fortified with the glucuronide standards. Linear regression analysis of the peak area as a function of the amount of *R*- and *S*-ketoprofen glucuronides in the range 1.25–25.0 μg ml^{-1} yielded a coefficient of correlation better than 0.999. The equation for the regression line was $y = 418\ 340x - 67\ 230$ for *R*-ketoprofen glucuronide and $y = 449\ 840x - 55\ 205$ for *S*-ketoprofen glucuronide, where y is the peak area (arbitrary units) and x the concentration (μg ml^{-1}). Repeatability was calculated for five replicate injections of 10.0 μg ml^{-1} *R*- and *S*-ketoprofen glucuronides, and R.S.D. values were respectively 1.2 and 1.4%. Recovery from microsomal suspensions was 85% and the limit of detection was 10 ng for each glucuronide injected (signal-to-noise ratio = 3).

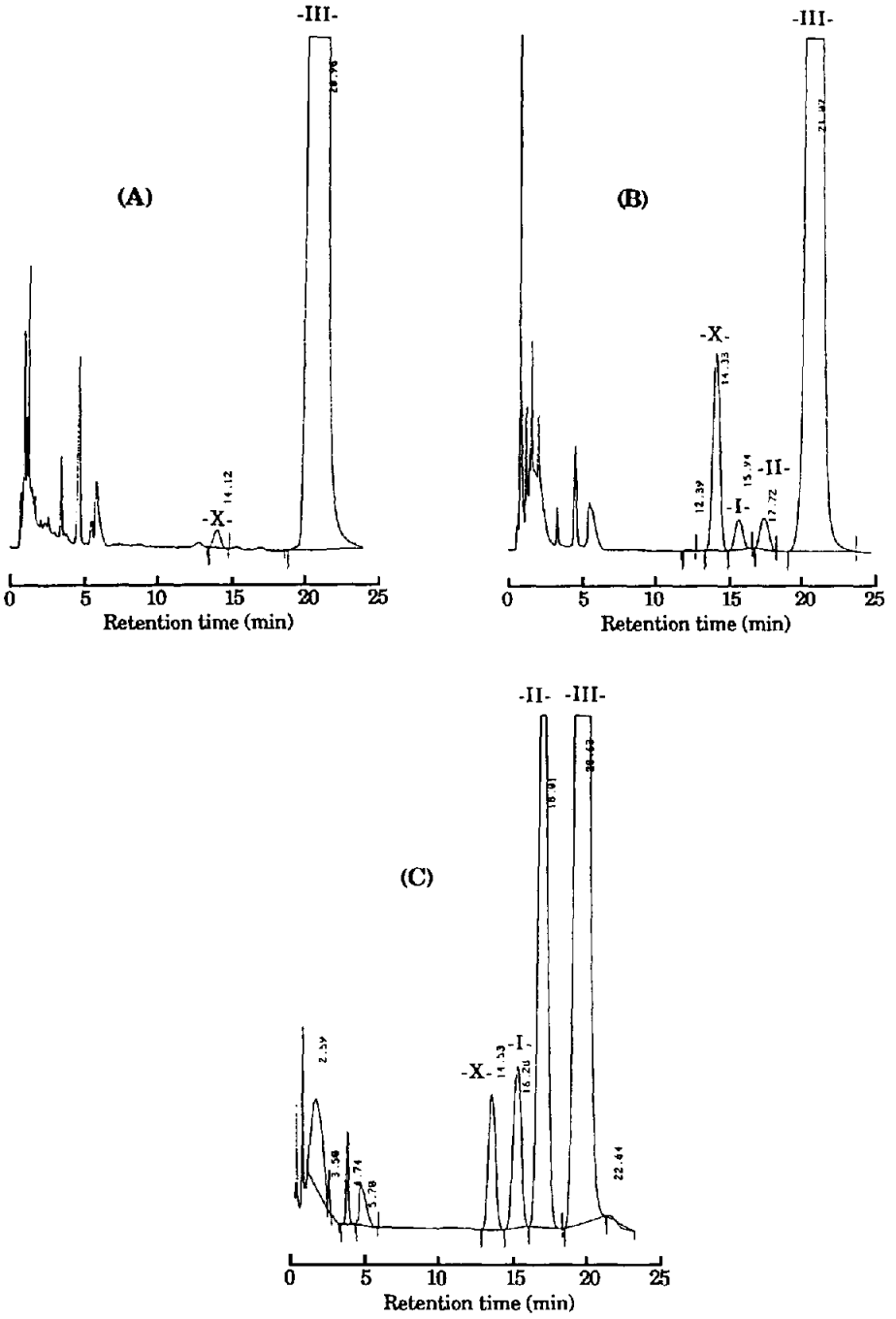


Fig. 3. Typical chromatograms obtained from microsomal incubation of ketoprofen with UDP-glucuronic acid and after liquid–solid extraction. *S*-ketoprofen glucuronide (I), *R*-ketoprofen glucuronide (II), *R,S*-ketoprofen (III), (X) unknown compound. (A) control: microsomal suspension spiked with *R,S*-ketoprofen but without incubation; (B) 20-min incubation with human liver microsomes; (C) 20-min incubation with dog liver microsomes. The chromatographic conditions were: Superspher (4 μm) 100 RP-18 end capped 125 × 4 mm I.D. column eluted with acetonitrile–10 m*M* tetrabutylammonium bromide in 1 m*M* potassium phosphate adjusted to pH 4.3 (30:70, v/v); UV detection at 254 nm.

The chromatograms obtained from the microsomal samples were free of interfering and late cluting peaks (Fig. 3), which allowed a frequency of analysis up to 2 samples per hour.

This technique was then applied to the *in vitro* determination of the glucuronidation of *R,S*-ketoprofen in hepatic microsomes from various animal species (dog, rat, rabbit) and man (Fig. 4). The enzymatic formation of ketoprofen glucuronides was linear with respect to time (up to 30 min) and protein concentration (in the range 0.1–0.5 mg). Of all the species considered, dog presented the highest specific activity. It could glucuronidate the *S*-enantiomer 4 times faster than the *R*-antipode. These data indicate that the glucuronidation of ketoprofen is stereoselective in dog. We previously found that dogs were generally very active in glucuronidating the NSAIDs belonging to the class of profens such as pirprofen, flurbiprofen and the chemically related compound 2-phenylpropionic acid [24]. On the other hand, dog is well known for undergoing stereoselective glucuronidation of drugs [25].

Rat liver microsomes could glucuronidate ketoprofen at a rate and with an enantiomeric ratio of *ca.* 1, which is similar to that obtained with human liver microsomes (Fig. 4). Such a ratio has also been reported by Volland and Benet [7]. In this respect, rat appears to present an animal model suitable to study the *in vitro* glucuronidation of ketoprofen in man. However, from *in vivo* experiments in rats it was concluded that *R*-ketoprofen was almost completely inverted into the *S*-enantiomer *via* the formation of an acyl-CoA derivative, thus favoring the excretion of *S*-ketoprofen glucuronide [26,27]. On the other hand, rabbits glucuronidated the drug at the lowest extent (Fig. 4).

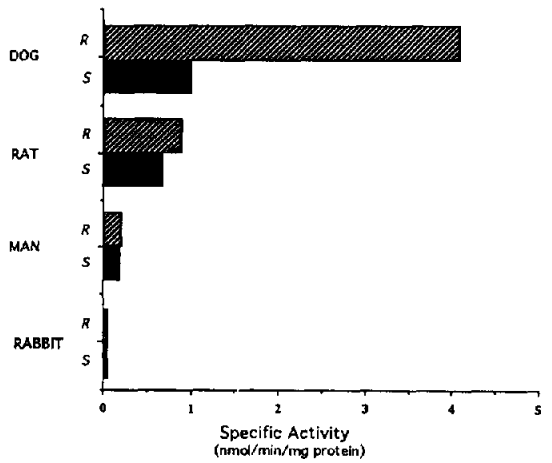


Fig. 4. Glucuronidation of *R*- and *S*-ketoprofen in man and animal species. Liver microsomes were incubated with 1 m*M* *R*- or *S*-ketoprofen and 3 m*M* UDP-glucuronic acid for 20 min at 37°C. The glucuronide formed were separated on the HPLC system, as described in Experimental. Values are the mean of 3 determinations on an individual microsomal sample.

4. Conclusions

A sensitive, reliable and accurate analytical method has been developed to investigate the *in vitro* glucuronidation of each enantiomer of ketoprofen in liver microsomes of various animal species and in man. This technique provides a valuable tool to study the stereoselectivity of the glucuronidation reaction. Preliminary results indicate that this HPLC method can also be used to follow and quantitate the urinary excretion of the two diastereoisomers of ketoprofen glucuronide in man.

5. Acknowledgements

The authors thank Dr M. Abiteboul for the generous gift of ketoprofen enantiomers and Dr J.M. Ziegler for the determination of mass spectra. This work was supported in part by a grant from Institut National de la Santé et de la Recherche Médicale (INSERM, CRE No. 920308) and by the Association de Recherche sur la Polyarthrite.

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