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Note

Measurement of carprofen enantiomer concentrations in plasma and urine using L-leucinamide as the chiral coupling component*

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The measurement of drug enantiomer concentrations in biological fluids is often a difficult problem, which recently appeared to have been solved by the use of chiral stationary phases. However, the use of such columns has not met with the degree of success that was initially anticipated since the choice of mobile phase compositions is rather limited, resulting in a lack of flexibility, and because plasma and urine constituents, in addition to the drug of interest, may interact with the chiral column. Thus, chiral derivatization, resulting in the formation of diastereoisomers that are separable by normal- or reversed-phase high-performance liquid chromatography (HPLC), is now most often utilized for the quantification of enantiomers in biological samples. For the anti-inflammatory drug carprofen (Fig. 1), derivatization with α-methylbenzylamine was previously used to quan-

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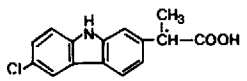

Fig. 1. Chemical structure of carprofen. The asterisk denotes the chiral carbon

tify the enantiomers in biological fluids. This derivatization procedure was first described by Stoltenborg et al. [1] and later improved by Lee et al. [2].

In the present study, we describe chiral derivatization of carprofen with L-leucinamide, after activation with ethyl chloroformate forming the mixed anhydride [3]. This reaction was introduced into the analysis of 2-arylpropionic acids by Björkman in 1985 [4] for the chiral derivatization of indoprofen and subsequently for ketoprofen [5]. Such a procedure, or modifications of it, was used for the assay of several non-steroidal anti-inflammatory drugs, such as ketoprofen [6], fenoprofen [7], tiaprofenic acid [8] and flurbiprofen [9]. We present here a sensitive and specific method for measuring *S*-(+)- and *R*-(−)-carprofen in plasma and urine.

EXPERIMENTAL

Chemicals and reagents

Racemic carprofen was obtained from Hoffmann-La Roche (Basle, Switzerland), and *S*-naproxen from Grünenthal (Stolberg, F.R.G.). (All samples and solutions containing carprofen were protected from light.) Ethyl chloroformate, triethylamine and L-leucinamide were purchased from Fluka (Buchs, Switzerland). All solvents were analytical grade, obtained from E. Merck (Darmstadt, F.R.G.). α-Methylbenzylamine enantiomers were purchased from EGA (Steinheim, F.R.G.).

Equipment

HPLC separations were performed using a DuPont preparative liquid chromatograph (DuPont, Wilmington, DE, U.S.A.) equipped with a Perkin Elmer 650-10 S fluorescence detector (Perkin Elmer, Überlingen, F.R.G.) for analytical measurements and a fraction collector and a DuPont 830 UV spectrophotometer (with a flow cell for preparative purposes). Optical rotations were measured with a Polartronic polarimeter (Schmidt Haensch, Berlin, F.R.G.). A vacuum evaporator (Rotavapor, Büchi, Flawil, Switzerland) was used to evaporate solvents on a preparative scale. This technique was utilized each time when solvent evaporation is mentioned unless other details are indicated.

Resolution of carprofen enantiomers via their diastereomeric salts

R-(+)-α-Methylbenzylamine (0.8 g) was dissolved in 4 ml of acetone and 1.8 g racemic carprofen was dissolved in 36 ml acetone. Without stirring, the α-methylbenzylamine solution was added slowly to the carprofen solution. This mixture was allowed to stand at room temperature, protected from light, for three days. The resulting precipitate was dried, again dissolved in acetone and allowed to stand for three days. The crystallization procedure was repeated and after the fourth crystallization the optical rotation was +13.15° (in methanol). A 250-mg

portion of the salt was dissolved in methanol. With stirring this solution was poured into a mixture of 20 ml of ice and 20 ml of 0.1 *M* hydrochloric acid. The mixture was kept cool for 1 h and the residue was separated and dried. The optical rotation for the carprofen enantiomer was +48° (in methanol).

A similar procedure was followed using *S*-(−)-α-methylbenzylamine as the chiral base for the second carprofen enantiomer. The enantiomeric purities (in % or calculated as the fraction of the product which exists as the enantiomer of interest minus the fraction of the other enantiomer (=*e*) according to Bähr and Theobald [10]) of the products were 95.1% (*e*=0.90) for *S*-carprofen and 95.4% (*e*=0.91) for *R*-carprofen.

Resolution of carprofen enantiomers by semipreparative HPLC resolution of their diastereomeric α-methylbenzylamine derivatives and subsequent hydrolytic cleavage

R/S-Carprofen (1 mmol) was dissolved in 25 ml of toluene, then 2.5 ml of thionyl chloride (freshly distilled over linseed oil) were added and the mixture was refluxed for 20 min. The solvent and the excess thionyl chloride were first evaporated using a vacuum evaporator and then under dried nitrogen to remove the remaining traces of thionyl chloride and solvent (yield: 82.9%, $C_{15}H_{11}NOCl_2$, MW 292.2).

Carprofen chloride (0.5 mmol) was then dissolved in 12 ml of dichloromethane. To this 0.4 ml of *S*-(−)-α-methylbenzylamine dissolved in 3 ml of dichloromethane was added. The mixture was refluxed for 10 min and the solvent evaporated. The residue was dissolved in diethyl ether. This solution was washed twice with 50 ml of 0.2 *M* hydrochloric acid and then three times with water. After washing, the solvent was evaporated and the residue dissolved in a mixture of dichloromethane–10% methanol. This solution was dried with sodium sulfate overnight. The diastereomeric carprofen amides were obtained after evaporation of the solvent (yield: 91%, $C_{23}H_{21}N_2OCl$, MW 376.9).

Resolution of the amides was achieved on a preparative HPLC column filled with Zorbax Sil (250 mm×21.2 mm, 7 μm particle size, DuPont), equipped with a pre-column (40 mm×4.6 mm) filled with LiChrosorb Si 100 (30 μm). A mixture of cyclohexane–dichloromethane–tetrahydrofuran (10:10:1, v/v) was used. The system was operated at a pressure of 5.5 MPa (55 bar) at room temperature, resulting in a medium flow-rate of 15 ml/min. The diastereomeric amides were detected at 285 nm (UV absorption) and collected separately. The solvent was then evaporated under vacuum and the residue dried over phosphoric pentoxide.

Recrystallization from acetone was carried out. Hydrolysis was performed by dissolving the amide in toluene (0.5%), adding the same volume of 20% hydrochloric acid and heating this mixture to 75°C for 60–120 min. The toluene layer was removed. The carprofen enantiomer was then extracted from the pH 2–3 aqueous phase with dichloromethane and the solvent evaporated. The toluene layer was evaporated as well. The residues were combined and the compound recrystallized in methanol. The enantiomeric purities were 95.1% (*e*=0.90) for *S*-carprofen and 97.1% (*e*=0.94) for *R*-carprofen.

Extraction of carprofen enantiomers from biological material

Plasma. Plasma (0.2 ml) was pipetted into a screw-capped centrifuge tube. A 1-ml volume of pH 5 buffer (citrate) was added and the mixture extracted with 5 ml of diethyl ether–dichloromethane (4:1, v/v). The glucuronide conjugates of carprofen were quantified by cleavage prior to extraction and derivatization with L-leucinamide. To 0.2 ml of plasma were added 10 μg of naproxen as internal standard and 0.2 ml of 1 *M* sodium hydroxide solution; the mixture was allowed to stand at room temperature for 20 min protected from light. The solution was then neutralized with 1 *M* hydrochloric acid and adjusted to pH 5 by adding 1 ml of pH 5 buffer. After extraction with diethyl ether–dichloromethane as described above and evaporation to dryness under nitrogen, the derivatization procedure was performed as given below. The resulting measured concentrations are the sum of both conjugated and unconjugated enantiomers in plasma.

Urine. Urine (1 ml) was adjusted to pH 5 and the enantiomers were extracted and derivatized as described for plasma. To determine the concentrations of glucuronides, 0.1 ml of urine was treated with 0.2 ml of 1 *M* sodium hydroxide for 20 min (after addition of naproxen). After neutralization with 1 *M* hydrochloric acid and subsequent adjustment to pH 5, the enantiomers were extracted with diethyl ether–dichloromethane (4:1). The organic layer was evaporated to dryness under nitrogen.

Carprofen glucuronide concentrations in plasma and urine were also determined by enzymatic hydrolysis where 100 μl of pH 5 buffer and 10 μl of β-glucuronidase were added to 100 μl of biological sample which was incubated overnight at room temperature.

Derivatization procedure

To the plasma or urine residues obtained, 50 μl of toluene were added and evaporated under nitrogen to remove traces of water. The residue was then dissolved in 100 μl of 50 m*M* triethylamine in (dried) acetonitrile; after 1 min, 50 μl of a 60 m*M* solution of ethyl chloroformate in dried acetonitrile and after another minute 50 μl of a solution of L-leucinamide hydrochloride (1 *M*) and triethylamine (1 *M*) in methanol were added. After 3 min the reaction was stopped by the addition of 0.2 ml of 0.25 *M* hydrochloric acid. The formed diastereomeric amides were then extracted with 4 ml of ethyl acetate. After evaporation of ethyl acetate, the residue was reconstituted in methanol or mobile phase.

Chromatographic separation of the diastereomeric L-leucinamide derivatives by reversed-phase HPLC

Separation of the diastereomeric derivatives was achieved on an octadecyl silane column (Zorbax ODS, DuPont) using 10 m*M* phosphate buffer pH 6.5–acetonitrile (45:55, v/v) as the mobile phase. Separation took place at a flow-rate of 1 ml/min at ambient temperature (medium pressure 15 MPa). The derivatives were detected by measuring their fluorescence at 285/345 nm.

Linearity, reproducibility and detection limits

The linear relationship between measured peak areas and spiked concentrations was investigated up to 175 μg racemate per ml plasma and 500 μg racemate

per ml urine (0.1-ml aliquots for sample work-up). The intra- and inter-day coefficients of variation were estimated for four different concentrations of carprofen racemate in plasma (10, 0.5, 0.1 and 0.04 μg/ml) and urine (150, 20, 1 and 0.1 μg/ml). The minimum quantifiable concentration (defined as three times baseline noise) was characterized by preparing and analyzing plasma and urine standards containing very low concentrations of carprofen.

Application of the method in a clinical study

One healthy female volunteer was administered 150 mg *R*/*S*-carprofen orally. Blood samples (5 ml) were taken before and 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 10, 15, 24 and 30 h after drug administration. Blood, collected in heparin-treated tubes, was cooled in ice immediately and centrifuged at 4 °C. Plasma was separated and adjusted to acidic pH by addition of 85% phosphoric acid (10 μl per ml of plasma) and frozen until analysis. Urine was collected in intervals. The urine samples were adjusted to pH 3 by adding phosphoric acid and also frozen until analysis. Unconjugated and conjugated carprofen enantiomer concentrations were assayed in plasma and urine samples. Conjugated carprofen concentrations were calculated as the measurement after chemical hydrolysis minus the measurement of unconjugated carprofen concentrations before hydrolysis.

RESULTS AND DISCUSSION

The enantiomeric purities obtained via formation of the diastereomeric salts from fractionated crystallization and obtained via the diastereomeric derivatives from semipreparative HPLC separation were greater than 95%. After resolution of the enantiomers, the elution order was characterized, and the extraction and derivatization behaviors of the two enantiomers were investigated for possible differences. Retention times for naproxen, *R*-(−)-carprofen and *S*-(+)-carprofen were 6.6, 9.4 and 11.0 min with corresponding capacity factors (k') of 5.5, 8.3 and 9.9. With the described mobile phase, which is similar to that used by Björk-

Fig. 2. Representative chromatograms for the separation of the diastereomeric carprofen derivatives in plasma samples 1 h after administration of 150 mg racemic drug. (a) Plasma concentrations: *R*-carprofen 2.9 μg/ml and *S*-carprofen 3.1 μg/ml. (b) Blank plasma sample. (c) Blank urine sample. i.st. = internal standard, naproxen.

man [4] for the resolution of indoprofen enantiomers, a separation factor of 1.19 and a resolution factor of 2.73 were achieved for the derivatives of the carprofen enantiomers. Fig. 2 depicts chromatograms for the derivatives after extraction from a plasma sample, blank plasma and blank urine.

The extraction yield was determined over the pH range 1–8 and found to be highest at pH 5 (98%, compared with a methanolic solution of carprofen). No significant interference between the enantiomers during extraction and derivatization was detected by analyzing samples spiked with racemic drug, single enantiomers or different enantiomer combinations.

Comparing the yields from chemical and enzymatic hydrolysis, alkaline hydrolysis yielded slightly higher concentrations of both carprofen enantiomers. This can be explained as resulting from traces of acyl migration isomers of the β-1-O-acyl glucuronide, which are known to be formed under physiological conditions [11]. Such isomers are β-glucuronidase-resistant but susceptible to alkaline cleavage [11].

Linearity, reproducibility and detection limits

Calibration curves were linear up to 100 μg/ml racemate in plasma and 250 μg/ml racemate in urine ($r > 0.995$). For higher concentrations, the samples were diluted with plasma or urine, respectively. For the assay of unknown samples, a six-point calibration curve was established [e.g., $y = 0.174\ (\pm 0.006)x + 0.04\ (\pm 0.02)$; $r^2 = 0.997\ (\pm 0.002)$ for the *R*-enantiomer in plasma].

The intra-day coefficients of variation (C.V.) were 7.2% for *S*-carprofen and 6.3% for *R*-carprofen at a concentration of 500 ng/ml racemate in plasma samples (see Table I). The inter-day coefficients of variation did not exceed 12.5%. Minimum quantifiable concentrations for carprofen enantiomers were defined to be

TABLE I

PRECISION OF THE ANALYTICAL PROCEDURE FOR PLASMA AND URINE STANDARDS, INCLUDING THE INTRA-DAY COEFFICIENTS OF VARIATION AT DIFFERENT CARPROFEN CONCENTRATIONS

In each case, $n = 8$.

Concentration (μg/ml)		Coefficient of variation (%)	
Spiked	Found	*S*-Carprofen	*R*-Carprofen
Plasma			
0.040	0.0389	10.9	11.1
0.100	0.1012	8.9	8.1
0.500	0.499	7.2	6.3
10	9.98	5.9	5.4
Urine			
0.1	0.10	13.0	12.5
1	1.05	4.9	4.2
20	19.9	5.3	5.3
150	152	8.0	6.9

20 ng enantiomer per ml of plasma for a 0.2-ml aliquot and 50 ng enantiomer per ml of urine based on a 1-ml sample aliquot.

Concentrations of carprofen enantiomers and their conjugates in plasma and urine after oral dosage

After administration of 150 mg of racemic drug, maximum concentrations of 12.9 μg/ml for *S*-carprofen and 10.6 μg/ml for *R*-carprofen ($S > R$) were detected in plasma samples obtained from a healthy volunteer. The maximum concentration of glucuronide occurring in plasma was higher for the *R*-enantiomer than for the *S*-enantiomer (1.51 versus 1.25 μg equiv./ml). The concentration–time curves

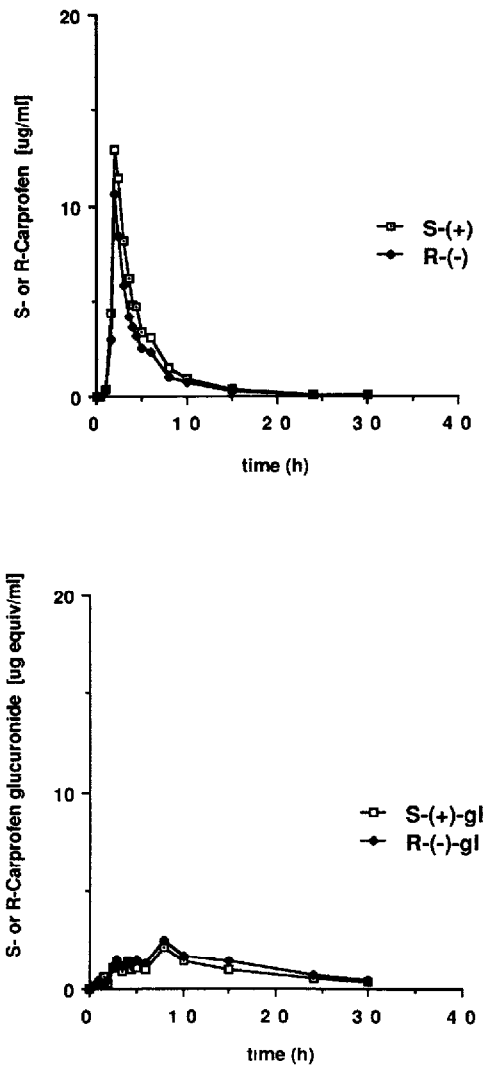

Fig. 3. Plasma concentrations of *S*- and *R*-carprofen in unconjugated form (upper plot) and conjugated form (lower plot) after dosage of 150 mg *R*/*S*-carprofen to a healthy young female volunteer.

for *S*- and *R*-carprofen and their respective glucuronide conjugates are depicted in Fig. 3.

The cumulative amount excreted in urine of unconjugated plus conjugated carprofen was 128 mg (85% of dose), with the renal clearance of unchanged enantiomers being very low (*R*, 0.27 ml/min; *S*, 0.16 ml/min) with a recovery of unconjugated carprofen of 1.25 mg (*R*, 0.64 mg; *S*, 0.61 mg). The corresponding values for the urinary recovery of conjugates were 60.5 mg for *R*-carprofen and 66.0 mg for *S*-carprofen, while the renal clearances of the diastereomeric glucuronides were 30.7 ml/min for *R*-carprofen and 41.1 ml/min for *S*-carprofen.

This suggests, in one subject, that the renal clearance of *R*-carprofen was higher than that of *S*-carprofen, whereas for the conjugates the renal clearance of *S*-glucuronide was higher than that of the *R*-glucuronide. Our results are basically in agreement with those of Stoltenborg et al. [1], who also found the excretion via urine to be higher for the *S*-enantiomer (unconjugated plus conjugated compound) and the plasma concentrations of the *S*-enantiomer to exceed those of the *R*-enantiomer. The areas under the concentration–time curves were – even when normalized to dose – considerably lower in the study of Stoltenborg et al. [1], probably because they measured blood concentrations, whereas we measured plasma concentrations.

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