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*Report*

# Enantiospecific High-Performance Liquid Chromatographic (HPLC) Determination of Baclofen and Its Fluoro Analogue in Biological Material

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A method is described for the quantification of baclofen enantiomers in biological material (urine, plasma, and cerebrospinal fluid). The samples were extracted by liquid–solid extraction using Sep-Pak C18 cartridges. The subsequent derivatization procedure contained two separate steps. (1) The butyl esters of the enantiomers were formed using butanolic hydrochloric acid (followed by ion-pair extraction of the intermediate products). (2) A chiral derivatization was then performed using *S*-(+)-naproxen chloride as reagent. *S*-(+)-Benoxaprofen chloride can also be used. The diastereomeric amides were separated by high-performance liquid chromatography (HPLC) on a silica gel column (mobile phase, *n*-hexane/dichloromethane/ethanol; detection, fluorescence measurement at 335/365 nm). The described procedure was also used for the quantification of the fluoro analogue of baclofen. Urinary excretion of baclofen enantiomers was investigated in two healthy volunteers after p.o. administration.

KEY WORDS: baclofen; fluoro analogue; enantiomers; enantiospecific determination; urinary excretion.

## INTRODUCTION

Baclofen [*R*/*S*-(±)-4-amino-3-(4-chlorophenyl)butyric acid; Fig. 1a] is a centrally acting antispastic agent (1). The enantiomers of this compound were found to have different properties, the R-enantiomer being about 100 times more active than the S-enantiomer (2). However, as in most cases, the racemate is marketed. Because the kinetic disposition of the two enantiomers may also be different, the investigation of the pharmacokinetic behavior of both enantiomers is desirable. A high-performance liquid chromatographic (HPLC) procedure for the determination of baclofen enantiomers on chiral stationary phases was already described by Smith and Pirkle (3), who showed the chromatographic separation only, but did not quantify the enantiomers in biological material. Weatherby *et al.* (4) used a chiral mobile phase in order to separate the tritium-labeled enantiomers, which also cannot be used for pharmacokinetic routine analyses. Wuis *et al.* (5) developed a stereoselective analytical method based on the derivatization of baclofen with a chiral adduct of *o*-phthaldialdehyde. Again, applicability to pharmacokinetic studies has not yet been reported.

Recently, benoxaprofen and naproxen chloride (Figs. 2a and b) were described as chiral derivatization reagents (6,7). In the present paper, the use of these acyl chlorides for the derivatization of baclofen and its fluoro analogue (Figs. 2a and b) and the high-performance liquid chromatographic separation of the derivatives are described. An assay method for biological material was worked out based on the derivatization with naproxen chloride. Naproxen was preferred in spite of its lower fluorescence intensity as it is marketed and therefore easily accessible as the pure enantiomer. The method was tested for suitability in routine analyses in plasma, urine, and cerebrospinal fluid.

Furthermore, the same analytical method was applied for the determination of the fluoro analogue of baclofen (Fig. 1b), which was developed under the assumption of having fewer side effects in the therapeutic range (8).

## MATERIALS AND METHODS

### Instruments

A Beckman HPLC pump (Beckman Instruments, Palo Alto, Calif.) equipped with a Shimadzu RF-530 fluorescence detector (Shimadzu, Kyoto, Japan) was used for the HPLC separations. For the gas chromatographic analyses a Perkin Elmer F22 gas chromatograph was used (Perkin Elmer, Überlingen, F.R.G.). The thin-layer chromatographic determinations were performed using a Linomat III (Camag, Muttenz, Switzerland) for the application of solutions on the HPTLC plates and a KM3 chromatogram spectrophotometer (Zeiss, Oberkochen, F.R.G.).

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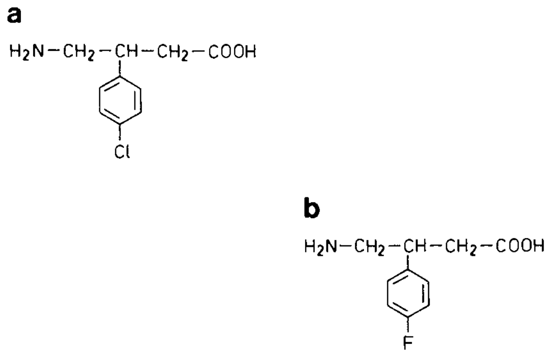

**Fig. 1.** Structural formulas of baclofen (a) and its fluoro analogue (b).

## Chemicals

Racemic baclofen, the fluoro analogue CGP 11.130 [*R*/*S*-4-amino-3-(4-fluorophenyl)butyric acid, as hydrochloride], and their enantiomers (as hydrochlorides) as well as the proposed internal standard, the "dichloro" derivative of baclofen [*R*/*S*-4-amino-3-(2,4-dichlorophenyl)butyric acid] were supplied by Ciba-Geigy (Wehr, F.R.G., and Basel, Switzerland). Solvents (analytical grade for extractions and LiChrosolv for HPLC), thionyl chloride, and decyl hydrogen sulfate sodium salt (analytical grade) were from E. Merck (Darmstadt, F.R.G.). Sep-Pak C18 liquid–solid extraction cartridges were purchased from Waters (Eschborn, F.R.G.). *S*-(+)-Naproxen was kindly donated by Grünenthal (Stolberg, F.R.G.). *R*/*S*-Benoxaprofen was a gift of Eli Lilly (Bad Homburg, F.R.G.). D-2-Octanol was purchased from Ega-Chemie (Steinheim/Albuch, F.R.G.). The enantiomers of tranylcypromine [enantiomeric purities—1S,2R-(+)-trans = 97.8% and 1R,2S-(−)-trans, = 98.1%; determined after derivatization with (+)-α-methoxy-α-trifluoromethylphenylacetic acid and gas chromatographic (GC) analysis as described previously (6)] were kindly donated by Röhm Pharma (Weiterstadt, F.R.G.).

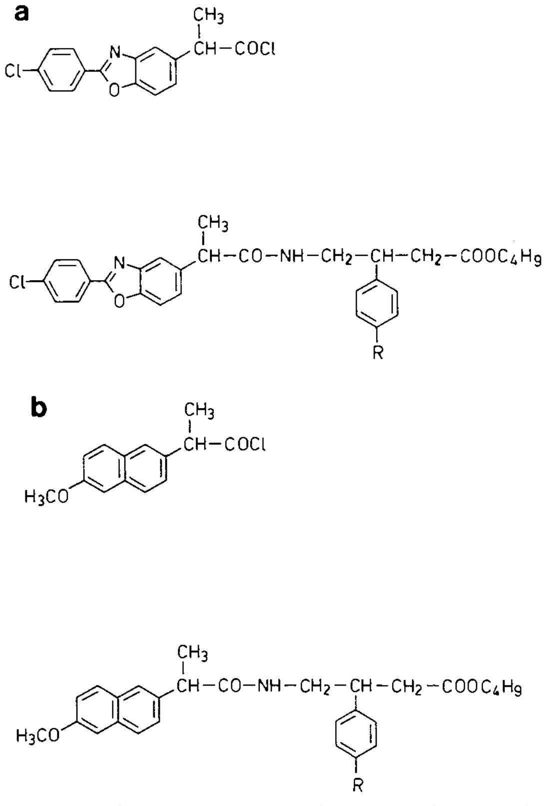

**Fig. 2.** (a) Structural formulas of benoxaprofen chloride and its derivatization product after having performed both derivatization steps. For baclofen R = Cl; for the fluoro analogue R = F. (b) Structural formulas of naproxen chloride and its derivatization product after having performed both derivatization steps. For baclofen R = Cl; for the fluoro analogue R = F.

## Synthesis of Acyl Chlorides

### *Naproxen Chloride (According to Refs. 7 and 9)*

Five hundred milligrams of naproxen was dissolved in 50 ml of dried toluene. After slowly adding 5 ml of thionyl chloride (freshly distilled over linseed oil) the solution was refluxed for 60 min in a darkroom. Then the solution was evaporated to dryness in vacuum. After being kept over potassium hydroxide under vacuum (over night), the crystalline residue was used without further purification. All these procedures were carried out under protection from light.

### *Benoxaprofen Chloride (According to Refs. 6 and 10)*

After resolution of the benoxaprofen enantiomers by preparative HPLC after derivatization with α-methylbenzylamine and subsequent hydrolysis, the acyl chloride of the pure enantiomer was formed. Benoxaprofen chloride was synthesized in the same way as described for naproxen. Refluxing benoxaprofen and thionyl chloride in toluene takes 1 hr. As benoxaprofen is not as photounstable as naproxen, precautions such as protection from daylight are not necessary.

## Determination of the Enantiomeric Purities of Reagents and Reference Compounds

### S-*(+)-Naproxen Chloride*

The determination was performed by two different methods according to Ref. 9. The gas chromatographic determination of the *S*-(+)-naproxen-D-2-octyl ester resulted in an enantiomeric purity of 99.05% (GC conditions: OV17 packed column; FID; temperatures—inj., 245°C; column, 260°C; det., 270°C). Only slightly different results were obtained by thin-layer chromatography (TLC) of the *S*-naproxen derivatives of (+)- or (−)-tranylcypromine. Chromatographic conditions were as follows: silica-coated HPTLC plate; mobile phase, toluene/dichloromethane/tetrahydrofurane (50:30:10; ammonia was added in a separate beaker). The values found using this method were 98.5 and 98.7% using (+)- and (−)-tranylcypromine, respectively.

### S-*(+)-Benoxaprofen Chloride*

Tranylcypromine enantiomers were used for the evaluation of the enantiomeric purity of the benoxaprofen enantiomers (as acyl chlorides) by HPLC as described previously (6). The enantiomeric purity of the *S*-(+)-enantiomer was found to be 97.1%.

### *Baclofen and Its Fluoro Analogue*

The enantiomeric purity was determined indirectly, by derivatization with *S*-(+)-naproxen chloride according to the method described here. In each case 5 μg of the enantiomer was esterified and reacted with naproxen chloride. The diastereomeric amides were immediately analyzed by HPLC. Calculations of *e* as a measure of the excess of one enantiomer toward the other were performed according to Bähr and Theobald (11).

## Preparation of the Pure Derivatization Products

### *Formation of the Butyl Ester*

To 1.0 g of baclofen or the fluoro analogue 15 ml of a solution of hydrochloric acid (3 *N*) in butanol (prepared with HCl gas) was added. The mixture was refluxed for 30 min under anhydrous conditions at 105°C. With cooling, the ester crystallized as hydrochloride from the reaction mixture. The crystals were separated and dried over phosphorus pentoxide. The purity of the ester was sufficient without further recrystallization.

### *Formation of the* S-(+)*-Naproxen Amide*

Seventy-five milligrams of *S*-(+)-naproxen chloride was dissolved in 10 ml of dried dichloromethane and about 0.5 g of sodium hydrogen carbonate (water-free) and 20 ml of a solution of the butyl ester hydrochloride (of baclofen or the fluoro analogue) in dried dichloromethane (15 mg/ml) was slowly added. The mixture was stirred for 5 hr at ambient temperature. The hydrogen carbonate was filtered off. Then the organic phase was washed with 1 *N* hydrochloric acid (30 min) and subsequently with 1 *N* sodium hydroxide (3 hr) and then water until the pH value was neutral again. After the removal of water with water-free sodium sulfate, the solvent was evaporated under vacuum. The residue was readily soluble in a mixture of ethanol and water (10:1). When the solution was cooled and water added dropwise, the product crystallized out and was filtered off. After drying over phosphorus pentoxide, the derivatization product could be used without any further purification.

### *Product Characterization*

*Baclofen Derivative.* Total yield, 69%; m.p., 89°C. Enantiomeric composition of the product S/R (referred to baclofen): 1.7, IR ($cm^{-1}$): 1650 ($>C{=}O$, amide), 1730 ($>C{=}O$, ester). Elementary analysis ($C_{28}H_{32}ClNO_4$): C, 69.73; H, 6.79; N, 2.81% (calcd: C, 69.77; H, 6.60; N, 2.81%). $^1H$-NMR (solvent, $CDCl_3$; standard, TMS): $\delta$ = 0.84 (t, 3H, $-CH_2\text{-}CH_3$); 0.85 (t, 3H, $-CH_2\text{-}CH_3$); 1.22 (m, 2H, $-CH_2\text{-}CH_3$); 1.43 (m, 2H, $-O-CH_2\text{-}CH_2\text{-}$); 1.53 (d, 3H, $CH_3\text{-}CH<$); 1.56 (m, 1H, $-CH_2\text{-}CH-CH_2\text{-}$); 2.49 (q, 1H, $>CH\text{-}CO\text{-}$); 3.17–3.61 (m, 4H, $\text{-}NH-CH_2\text{-}$, $-CH_2\text{-}COO$); 3.92 (t, 2H, $\text{-}O-CH_2-CH_2\text{-}$); 5.20 (m, 1H, -NH-); 6.76–7.68 (m, 10H, aromatic H). Because of the diastereomeric character of the compound, a split of some proton signals was observed ($\delta$ = 0.84, 0.85).

*Derivative of the Fluoro Analogue.* Total yield, 67%; m.p., 65°C. Enantiomeric composition of the product S/R (referred to the fluoro analogue): 0.8, IR ($cm^{-1}$): 1650 ($>C{=}O$, amide), 1730 ($>C{=}O$, ester). Elementary analysis ($C_{28}H_{32}FNO_4$): C, 71.99; H, 6.96; N, 3.01% (calcd: C, 72.24; H, 6.93; N, 3.01%), NMR: The NMR of the derivatization product of the fluoro analogue was identical to that one of the baclofen derivative with the exception of the $\delta$ = 6.76–7.68 scale, referring to the aromatic protons.

## Analytical Procedure for the Enantiomers of Baclofen and Its Fluoro Analogue

### *Extraction of Urine Samples and Derivatization Steps*

The samples were purified by liquid–solid extraction-using SepPak C18 cartridges. The C18 columns were preconditioned with 5 ml of methanol and 4 ml of 0.25 *M* hydrochloric acid. One hundred microliters of urine was then extracted using the cartridge. The urine pipette was rinsed with 100 μl of 0.25 *M* hydrochloric acid, which was also applied onto the cartridge, as well as 20 μl of an aqueous solution containing 1 μg of the internal standard, the "dichloro" derivative. The cartridge was then washed with 3 ml of 0.25 *M* HCl and 200 μl of methanol. Subsequently, baclofen or its analogue were washed out with 0.7 ml of methanol. The solution was collected in a screw-capped centrifuge tube and evaporated in a vacuum centrifuge. One milliliter of 0.01 *M* sodium decyl hydrogen sulfate solution in water and 5.5 ml of dichloromethane containing 5% of pentanol were added to the residue. The tubes were vortexed briefly, then shaken on a horizontal mixer for 30 min and centrifuged (10 min, 4000 rpm). The upper (aqueous) layer was removed. Then 5 ml of the organic layer was transferred into a second centrifuge tube. The solvent was evaporated in a vacuum centrifuge. To remove traces of water, 200 μl of toluene was added and evaporated. After the addition of 1 ml of 3 *N* butanolic hydrochloric acid, the free carboxyl group was esterified by heating the mixture to 105°C for 20 min. Afterward the liquid was evaporated under nitrogen at the same temperature. About 20 mg of sodium carbonate (without crystal water) and 200 μl of reagent solution [3 mg of *S*-(+)-naproxen or, e.g., *S*-(+)-benoxaprofen chloride in 10 ml dried ethylacetate] were then added. Formation of the diastereomeric amides was performed at room temperature by shaking the tubes moderately for 60 min. After centrifugation 20 μl of the resulting solution was injected directly into the liquid chromatograph.

### *Analysis of Plasma Samples*

In general the extraction and derivatization steps were identical to those for urine with a few exceptions: 1.0 ml of plasma was used, and 20 μl of an aqueous solution (25 μg/ml) of the internal standard was added (=500 ng). After esterification 1 ml of $2 \times 10^{-4}$ *M* sodium decyl hydrogen sulfate solution and 6.0 ml of dichloromethane were added. After shaking for 15 min and centrifugation (10 min, 4000 rpm) the upper (aqueous) phase was removed, the organic layer was evaporated, and the procedure was continued as described for urine.

### *Analysis of Cerebrospinal Fluid (CSF)*

The extraction and derivatization steps were performed in exactly the same way as for urine with one exception: a

500-μl sample was needed, which was acidified with 100 μl of 1 *M* HCl. Twenty microliters of a 10 μg/ml solution of the internal standard was added.

### *HPLC Analysis*

Twenty microliters (for urine samples) or 50 μl (for plasma and CSF) was injected (total sample volume, 200 μl) on a silica gel column (Zorbax Sil, 5 μm, 250 × 4.6 mm, DuPont, Wilmington, Del.). A mixture of *n*-hexane, dichloromethane, and ethanol (100:9.4:1.6, v/v) was used as the mobile phase. The chromatographic separation was performed at ambient temperature, at a flow rate of 1.5 ml/min, which results in an average pressure of 2.1 MPa (300 psi). The naproxen derivatives could be detected by measuring the fluorescence (excitation, 335 nm; emission, 365 nm). The retention times of the derivatives were 14 min for the R- and 15 min for the S-enantiomer for both baclofen and the fluoro analogue and 11 and 12 min for the internal standard.

The benoxaprofen derivatives were also separated on a silica gel column, using a different mobile phase [dichloromethane, *n*-hexane, *n*-propanol (75:25:2.45, v/v)]. The HPLC separation was also carried out at ambient temperature and a flow rate of 1 ml/min, resulting in a pressure of 4.2 MPa (600 psi). The detection wavelengths for the benoxaprofen derivatives were 313 nm as the excitation and 365 nm as emission wavelength. The retention times were 19 min for the *S*-(+)-benoxaprofen derivative of the S- and 22 min for that of the R-enantiomer.

The coefficient of variation was determined for three different concentrations of drug (4.5, 0.9, and 0.15 μg racemic drug/100 μl of urine; 1 μg/ml for plasma and CSF) with and without addition of the internal standard. In order to quantify the amount of enantiomer recovered after extraction from biological material and derivatization with naproxen chloride, the synthesized product was used as the reference. The peak height obtained after direct injection of different amounts of the pure product was compared to the peak heights of plasma and urine standards. The peak heights could be used for the calculation of enantiomer concentrations in unknown samples up to an amount of at least 1.6 μg/peak, if naproxen was used for derivatization. The concentrations were corrected with respect to the enantiomeric impurity of the reagent according to Hermansson and von Bahr (12).

### *Enantiomeric Stability of Baclofen and Its Fluoro Analogue in Biological Material*

Spiked urine and plasma samples were used to evaluate the long-term stability of the enantiomers of both compounds. The sample concentrations were 20 μg drug/ml urine and 2 μg/ml plasma for the racemates and half the concentrations for each of the enantiomers, resulting in the same enantiomer concentrations. The enantiomer concentrations were investigated after 4 and 12 months of storage at −20 and +4°C and compared to freshly prepared standards using the described method. No decomposition or racemization was observed.

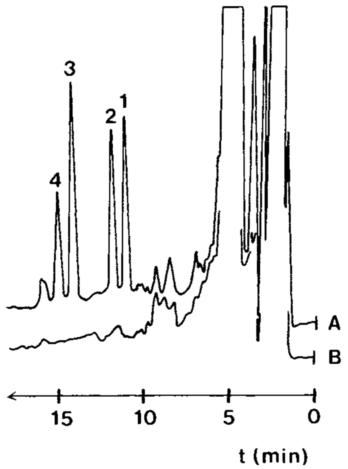

Fig. 3. Chromatograms of urine samples obtained from volunteer 2 after extraction, formation of the butyl ester, and derivatization with naproxen chloride. (A) Sample collected 0–2 hr after p.o. administration of 20 mg racemic baclofen. Enantiomer concentrations found: S-(+), 3.01 μg/ml; R-(−), 4.98 μg/ml. (B) Blank urine; derivatization products (1, 2) of the internal standard; (3) of *R*-(−)-baclofen, and (4) of *S*-(+)-baclofen.

### *Application of the Method*

The urinary excretion of baclofen enantiomers in two healthy volunteers (after p.o. administration of 20 mg racemic drug) was determined. Furthermore, the urinary excretion of the enantiomers of the fluoro analogue (dose, 100 mg racemic drug) was investigated in one of the volunteers. Urine was collected at 2-hr intervals up to 12 hr, then at 12–16 and 16–24 hr.

## RESULTS AND DISCUSSION

A chromatogram of baclofen enantiomers after extraction from a urine sample and derivatization using naproxen chloride for the second step is given in Fig. 3. The derivatives of the fluoro analogue show the same chromatographic and fluorescence behavior. The separation factor α was 1.09, with a resolution factor *R* of 1.73, for baclofen and the fluoro analogue when naproxen was used and 1.08, with *R*

**Table I.** Enantiomeric Purities of Reagents and Reference Compounds

| | % | *e* |
|---|---|---|
| *R*-(−)-Baclofen | 98.60 | 0.972 |
| *S*-(+)-Baclofen | 97.99 | 0.960 |
| *R*-(−)-CGP 11.130 | 98.14 | 0.963 |
| *S*-(+)-CGP 11.130 | 97.98 | 0.960 |
| *S*-(+)-Naproxen | 99.05 | 0.981 |
| *R*-(−)-Benoxaprofen | 97.10 | 0.942 |

**Table II.** Coefficients of Variation (CV) for Baclofen in Urine, Plasma, and CSF ($N = 8$ in Each Case; the CV for the Fluoro Analogue Were Very Similar

| | CV (%) | |
|---|---|---|
| Concentration | Without internal standard | With internal standard |
| Urine samples (μg/100 μl) | | |
| 4.5 | 5.8 | 7.0 |
| 0.9 | 6.5 | 6.1 |
| 0.15 | 9.8 | 4.9 |
| Plasma samples (μg/ml) | | |
| 1.0 | 7.1 | 8.4 |
| CSF samples (μg/ml) | | |
| 1.0 | 6.3 | 4.9 |

being 1.88, when benoxaprofen was used for derivatization. The corresponding values for the derivatives of the internal standard, the dichloro derivative, were $\alpha = 1.09$ and $R = 1.63$. The enantiomeric purities of all investigated compounds are given in Table I.

The dichloro derivative provides a suitable internal standard. A small interfering peak, however, occurred sometimes with the same retention time as the diastereomeric derivative first eluted from the column. In such a case, only the second peak was used as a reference.

The time course of the reaction was the same for both enantiomers and for all the compounds. The reaction was almost complete after 45 min. Further reaction did not provide an additional increase in the peak areas. An enantioselective derivatization was not indicated.

The calibration curve of the naproxen method was linear up to the highest concentration tested, namely, 50 μg/ml in plasma, 500 μg/ml in urine, and 50 μg/ml in cerebrospinal fluid ($r = 0.999$). Sensitivity studies resulted in detection limits of 50 ng/ ml for plasma, 500 ng/ml for urine, and 50 ng/ml for cerebrospinal fluid samples (=4.5 ng/peak) using the sample volumes given in the experimental section. (All these concentrations are calculated as racemic drug.)

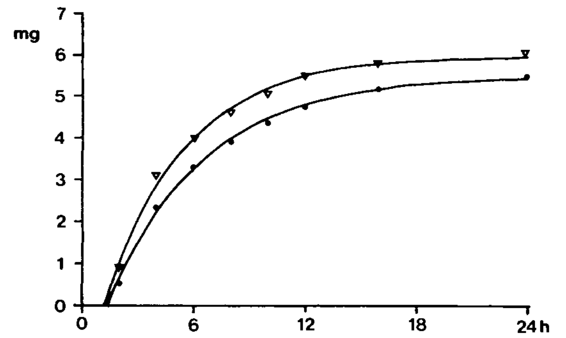

Fig. 4. Cumulative urinary excretion of baclofen enantiomers (mg) in volunteer 1 within 24 hr after p.o. administration of 20 mg racemic baclofen. Open triangles, *R*-(−)-baclofen; filled circles, *S*-(+)-baclofen. Curve fitted using a monoexponential function, which included a lag time.

**Table III.** Cumulative Amounts (mg) of the R- and S-Enantiomers of Baclofen and Its Fluoro Analogue in Urine Within 24 hr (Dosages: Baclofen, 20 mg Racemate; CGP 11.130, 100 mg Racemate)

| Volunteer No. | Drug | $A_e$ (R) | $A_e$ (S) |
|---|---|---|---|
| 1 | Baclofen | 6.1 | 5.5 |
| 2 | Baclofen | 7.8 | 6.7 |
| 2 | CGP 11.130 | 44.3 | 30.7 |

The coefficients of variation (CV) were always below 10%, and the use of an internal standard did not influence the CV significantly. The values are given in detail in Table II for baclofen. There was no difference between the two baclofen enantiomers. The variations found for the enantiomers of the fluoro analogue were similar to those of baclofen.

The yields from biological material for the complete analytical procedure were 89% for both baclofen enantiomers and 97% for the enantiomers of the fluoro analogue.

The method for the simultaneous determination of the enantiomers of baclofen and its fluoro analogue in biological material using the naproxen method was applicable to pharmacokinetic routine analyses. Analysis of urine samples from two healthy volunteers showed a significant difference between the excretion rates of the enantiomers. An example of the cumulative urinary excretion is shown in Fig. 4. The amounts of *R*- and *S*-baclofen excreted by two volunteers and of the *R*- and *S*-enantiomers of the fluoro analogue excreted by one volunteer within 24 hr after p.o. administration are given in Table III.

Although *S*-(+)-naproxen chloride is less fluorescent than benoxaprofen, it was selected because of its availability as the pure enantiomer. For measurement of plasma concentrations after a single-dose oral administration or lower i.v. doses, derivatization with a highly fluorescent marker (e.g., benoxaprofen chloride or naphthylethyl isocyanate) is required.

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