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ACTIVATED α-ALKYL-α-ARYLACETIC ACID ENANTIOMERS FOR STEREOSELECTIVE THIN-LAYER CHROMATOGRAPHIC AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF CHIRAL AMINES*

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SUMMARY

The separation of racemic benoxaprofen into the two benoxaprofen enantiomers by preparative high-performance liquid chromatography and the application of the activated enantiomers as derivatization reagents for the simultaneous stereoselective determination of chiral amines in biological material is described. Activated (+)- and (—)-benoxaprofen are both shown to be very sensitive and stable chiral fluorescence markers, applicable to thin-layer chromatography as well as to high-performance liquid chromatography.

INTRODUCTION

The chromatographic separation of optical isomers was already described by Kotake in 1951 [1] for the resolution of racemic amino acids using paper chromatography. In 1952 Dalgliesh postulated the so-called "three point attachment" necessary for the resolution of optical isomers [2]. Resolution of the enantiomers

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has been developed in two ways: (1) direct separation of enantiomers on an optically active stationary phase [3—5]; and (2) derivatization with a chiral reagent followed by chromatography of the diastereoisomers on a conventional stationary phase [6—8].

After the application of optically active drugs to humans or animals only small amounts of these substances can be found in biological material; sometimes the concentrations are in the picomole range or even lower. If the compounds do not have an intensive intrinsic fluorescence or strong chromophoric properties, it is necessary to have at one's disposal a chiral reagent that lowers the detection limit significantly. Beside adequate spectral properties, configurational purity and stability are demanded.

Up to now there has been no suitable fluorescence marker available for the determination of optically active amines in biological material that completely fulfils all these requirements. Chiral reagents used to form diastereoisomeric mixtures have some disadvantages: the tendency for racemization in a short period of time or insufficient fluorescent properties of the reagents themselves [9—12].

In the group of α-alkyl-α-arylacetic acids there are compounds that not only show a strong intrinsic fluorescence or ultraviolet (UV) absorbance or both, but also exist as racemates. For example, benoxaprofen [(*RS*)-2-(*p*-chlorophenyl)-α-methyl-5-benzoxazole acetic acid] turned out to be a fluorescence label well suited for the determination of amines and alcohols [13].

In this paper the separation of racemic benoxaprofen into the two benoxaprofen enantiomers and the application of the activated enantiomers as derivatization reagents for the simultaneous stereoselective determination of chiral amines in biological material will be described.

MATERIALS AND METHODS

Reagents and chemicals

Solvents (analytical grade), amphetamine, methamphetamine, thionyl chloride and thin-layer chromatographic (TLC) plates (silica gel) were obtained from E. Merck (Darmstadt, F.R.G.). Benoxaprofen was made available by Eli Lilly (Bad Homburg, F.R.G.), and tranylcypromine by Röhm Pharma (Weiterstadt, F.R.G.). α-Methylbenzylamine and α-methoxy-α-trifluoromethylphenylacetic acid were purchased from EGA (Steinheim, F.R.G.).

Apparatus

Melting points were obtained with a Büchi apparatus and are uncorrected. Infrared (IR) spectra were obtained in KBr disks with a Beckman Acculab 2 spectrophotometer.

Solutions were applied onto TLC plates using a Linomat III (Camag, Muttenz, Switzerland).

TLC plates were scanned with a chromatogram-spectrophotometer KM 3 (Carl Zeiss, Oberkochen, F.R.G.) and a recorder 56 (Perkin Elmer, Überlingen, F.R.G.).

A chromatograph LC 601 with a fluorescence detector 650-10 S (Perkin Elmer) and a preparative high-performance liquid chromatographic (HPLC) system 830 with a variable-wavelength UV spectrophotometer (DuPont,

Wilmington, DE, U.S.A.) were used for HPLC separations. Optical rotations were measured with a polarimeter Polartronic I (Schmidt + Haensch, Berlin). Gas chromatography (GC) was performed using a gas chromatograph F 22 (Perkin Elmer).

Separation of benoxaprofen enantiomers

Synthesis of benoxaprofen-α-methylbenzylamide. Benoxaprofen (600 mg, about 2 mmol) was dissolved in 50 ml of toluene. After slowly adding 5 ml of thionyl chloride (ca. a twenty-fold amount, freshly distilled over linseed oil) the solution was refluxed for 30 min and then evaporated to dryness. The crystalline residue was recrystallized from dichloromethane if necessary.

The benoxaprofen chloride was then dissolved in 50 ml of dichloromethane, and 10 ml of a solution of 1.5 ml of (*R*)-(+)-α-methylbenzylamine in 8.5 ml of dichloromethane were slowly added with stirring. After refluxing for 3 h the solution was washed first with 0.2 *M* hydrochloric acid, then with water, and dried over sodium sulphate. The solvent was evaporated and a white crystalline solid was recovered [13, 14]: m.p. 169°C (Lit. [14], 170°C), IR (cm^{-1}) 1640 (>C=O, amide). The solid gave one fluorescent spot (R_F 0.89) when examined by TLC in the solvent system chloroform—methanol—water—concentrated ammonia (70:30:5:1, v/v) [14], and two spots (R_F 0.28 and 0.16) in the solvent system toluene—dichloromethane—tetrahydrofuran (5:1:1, v/v), ammonia atmosphere.

Separation of the diastereoisomers using preparative HPLC. The diastereoisomeric mixture was separated into the two diastereoisomers: (*S*)-(+)-benoxaprofen-(*R*)-(+)-α-methylbenzylamide and (*R*)-(−)-benoxaprofen-(*R*)-(+)-α-methylbenzylamide. Chromatographic conditions were as follows: injection volume 2 ml (saturated solution of the diastereoisomeric mixture in mobile phase); guard column (DuPont) 50 mm × 4.6 mm I.D., packed with LiChrosorb Si 100 (30 μm); preparative HPLC column (DuPont) 250 mm × 21.2 mm I.D., stationary phase Zorbax-Sil, particle size 7 μm; mobile phase cyclohexane—dichloromethane—tetrahydrofuran (10:10:1, v/v); ambient temperature; flow-rate 15 ml/min (at a pressure of 5.0 MPa); detection by UV absorbance at 309 nm.

After collection of fractions of the column eluate, the solvent was evaporated to dryness. The residues were dried over phosphoric anhydride and recrystallized from acetone if necessary.

Hydrolysis of the amides. (*R*)-(−)-Benoxaprofen-(*R*)-(+)-α-methylbenzylamide (400 mg) was dissolved in 40 ml of toluene. After adding 40 ml of sulphuric acid (45%) the solution was refluxed for 1 h. The toluene phase, which contained only small amounts of benoxaprofen, was separated and evaporated to dryness. From the aqueous phase (*R*)-(−)-benoxaprofen was extracted at a pH value of 2—3 using dichloromethane. The dichloromethane phase was again evaporated to dryness. The residues from the dichloromethane and toluene phases were combined and dried over sodium sulphate. (*S*)-(+)-Benoxaprofen-(*R*)-(+)-α-methylbenzylamide was hydrolyzed in the same way. Benoxaprofen enantiomers can be recrystallized from methanol.

Activation of the benoxaprofen enantiomers to benoxaprofen chloride. The two enantiomers were activated to the respective acid chloride as described above.

Determination of the enantiomeric purity

In order to determine the enantiomeric purity of the benoxaprofen enantiomers, the enantiomeric purity of the substrates for derivatization (enantiomers of amphetamine, tranylcypromine, α-methylbenzylamine) must be known. The quantification of the enantiomeric purity of these amines was carried out by gas chromatography using activated α-methoxy-α-trifluoromethylphenylacetic acid, a reagent for the determination of the enantiomeric purity of alcohols and amines [15].

(+)-α-Methoxy-α-trifluoromethylphenylacetic acid (0.1 g) and 1 ml of distilled thionyl chloride were mixed and refluxed for 40 h. The excess thionyl choride was evaporated at 50°C in a stream of dry nitrogen and the residue dissolved in 10 ml of toluene. In each case 1 ml of this solution was added to about 10 mg of amine and the solution heated to 80°C for 1 h. The resulting amide was immediately assayed by gas chromatography.

Chromatographic conditions were as follows: injection volume 1 μl, split ratio 30:1; column OV-225, glass capillary, 25 m; carrier gas hydrogen; injection port temperature 230°C, oven 210°C, detector 250°C; flame-ionization detection. Under these conditions the enantiomeric purities were determined to be: 95.27% for (*S*)-(+)-amphetamine, 98.06% for (*S*)-(—)-tranylcypromine, 97.81% for (*R*)-(+)-tranylcypromine, 97.12% for (*S*)-(—)-α-methylbenzylamine, and 96.99% for (*R*)-(+)-α-methylbenzylamine.

After having obtained these results all the investigated amines were converted to the corresponding amide with (*S*)-(+)-benoxaprofen chloride or (*R*)-(—)-benoxaprofen chloride, in order to determine the enantiomeric purity of the benoxaprofen enantiomers themselves by HPLC (conditions described above).

General procedure for the determination of amines in biological material

Extraction and derivatization. One millilitre of plasma, 1 ml of 0.1 *M* sodium hydroxide solution and 3 ml of *n*-hexane were mixed in a centrifuge tube. After shaking (20 min) and centrifuging (10 min), 2 ml of the organic phase were transferred into another centrifuge tube and evaporated to dryness (using a vacuum centrifuge). Then 0.5 ml of reagent solution [1 mg of (*S*)-(+)-benoxaprofen chloride in 10 ml of dried dichloromethane] were added to the residue. The mixture was allowed to stand at room temperature for 30 min.

Chromatographic conditions. For TLC, the solutions were applied onto TLC plates (silica gel 60 without F_{254}) using a Linomat III. The volume applied was 10 μl, the band width 5 mm. Solvent systems were (I) toluene—dichloromethane—tetrahydrofuran (5:1:1, v/v), ammonia atmosphere, and (II) toluene—chloroform—tetrahydrofuran (5:4:1, v/v), ammonia atmosphere. Detection was by densitometric measurement of the fluorescence intensity using a chromatogram spectrophotometer KM 3; excitation wavelength was the 313-nm line of a mercury medium pressure lamp ST 41, slit 0.1 × 6 mm; emission, M 365 monochromatic filter; amplification, 1—10.

For HPLC, the injection volume was 10 μl. The analytical column (DuPont) was 250 mm × 4.6 mm I.D., with Zorbax-Sil (7 μm) as stationary phase. The mobile phase was cyclohexane—dichloromethane—tetrahydrofuran (5:1:1, v/v). Other conditions were: ambient temperature; flow-rate 1 ml/min (at a

pressure of 8.5 MPa); fluorimetric detection, excitation wavelength 312 nm, emission wavelength 365 nm.

RESULTS AND DISCUSSION

The results of all the investigations indicate that benoxaprofen enantiomers are well suited for the stereoselective determination of optically active compounds. The procedure for synthesizing the chiral reagent, which is described in this paper, can easily be carried out.

The liquid chromatographic separation of the synthesized diastereoisomeric mixture [(*RS*)-(±)-benoxaprofen-(*R*)-(+)-α-methylbenzylamide] using the preparative HPLC system described leads to diastereoisomers [(*S*)-(+)-benoxaprofen-(*R*)-(+)-α-methylbenzylamide and (*R*)-(—)-benoxaprofen-(*R*)-(+)-α-methylbenzylamide] of a high optical purity. Under the conditions described here retention times of 26 min for (*R*)-(—)-benoxaprofen-(*R*)-(+)-α-methylbenzylamide and 38 min for (*S*)-(+)-benoxaprofen-(*R*)-(+)-α-methylbenzylamide were observed. If 2 ml of a saturated solution of (*RS*)-(±)-benoxaprofen-(*R*)-(+)-α-methylbenzylamide in the mobile phase are injected, no tailing is observed and a baseline separation is always achieved. The difference in the chromatographic behaviour of the two diastereoisomers is more than sufficient for fractionated collection. The subsequent hydrolysis can be carried out in a short time without any detectable racemization.

The optical rotations ($[\alpha]_D^{20}$) of the enantiomers obtained [0.1% solution in chloroform—methanol (1:1, v/v)] were as follows: (*R*)-(—)-benoxaprofen —28.0°, (*S*)-(+)-benoxaprofen +27.5°, (*RS*)-(±)-benoxaprofen 0°.

Following GC and HPLC investigations the enantiomeric purity of the benoxaprofen antipodes was calculated to be 95.3% for (*S*)-(+)-benoxaprofen and 97.1% for (*R*)-(—)-benoxaprofen. When we tried using amino acid derivatives as optically active reagents, we could not obtain satisfactory results. In our experiments, the racemization rate of, for example, heptafluorobutyrylprolyl chloride (the reagent was proposed as a sensitive chiral marker for GLC

TABLE I

CHROMATOGRAPHIC PROPERTIES (TLC AND HPLC) OF FOUR DIFFERENT AMINES AFTER DERIVATIZATION WITH (*S*)-(+)-BENOXAPROFEN CHLORIDE

	R_F (TLC)		HPLC retention time (min)
	Solvent system I	Solvent system II	
(*S*)-(+)-Amphetamine	0.14	0.53	9.5
(*R*)-(—)-Amphetamine	0.21	0.59	8.0
(*S*)-(+)-Methamphetamine	0.27	0.65	11.5
(*R*)-(—)-Methamphetamine	0.33	0.65	10.5
(*R*)-(+)-α-Methylbenzylamine	0.28	0.56	6.7
(*S*)-(—)-α-Methylbenzylamine	0.16	0.45	10.8
(*R*)-(+)-Tranylcypromine	0.16	0.39	10.7
(*S*)-(—)-Tranylcypromine	0.21	0.45	9.0

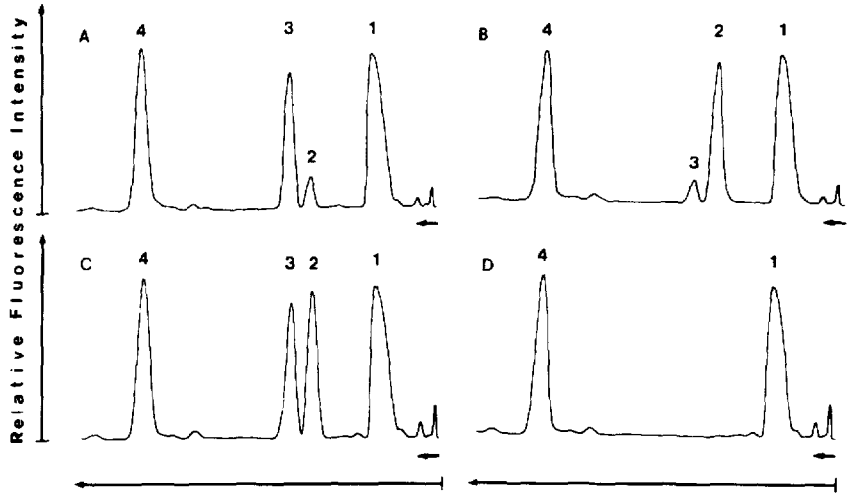

Fig. 1. Scans of thin-layer chromatograms (silica gel, solvent system I) of tranylcypromine (TCP) after extraction from plasma standards (5 μg/ml TCP) and derivatization with (*S*)-(+)-benoxaprofen chloride (1 = benoxaprofen, 2 = amide of (*R*)-(+)-TCP, 3 = amide of (*S*)-(—)-TCP, 4 = benoxaprofen chloride). (A) (*S*)-(—)-TCP; (B) (*R*)-(+)-TCP; (C) racemic TCP; (D) blank plasma.

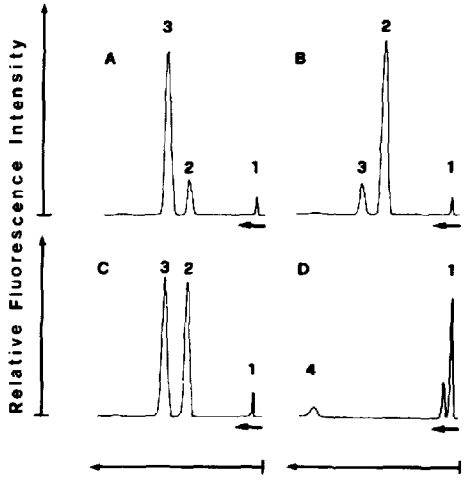

Fig. 2. TLC (silica gel, solvent system I) of α-methylbenzylamine (α-MBA) after extraction from plasma (5 μg/ml α-MBA) and derivatization with (*S*)-(+)-benoxaprofen chloride (1 = benoxaprofen, 2 = amide of (*S*)-(—)-α-MBA, 3 = amide of (*R*)-(+)-α-MBA, 4 = benoxaprofen chloride). (A) (*R*)-(+)-α-MBA; (B) (*S*)-(—)-α-MBA; (C) racemic α-MBA; (D) blank plasma.

[16]) was about 20%, although the experiments were carried out under mild conditions at very low temperatures (reaction temperature —70° C for 15 min).

The enantiomeric impurity of the benoxaprofen isomers themselves was found to be only 3% or 4%. If it is taken into account that the enantiomeric impurity of the (+)-α-methylbenzylamine that was used for the separation of the benoxaprofen isomers was about 3%, the racemization rate of (+)- or (—)-benoxaprofen during derivatization can be looked on as negligible.

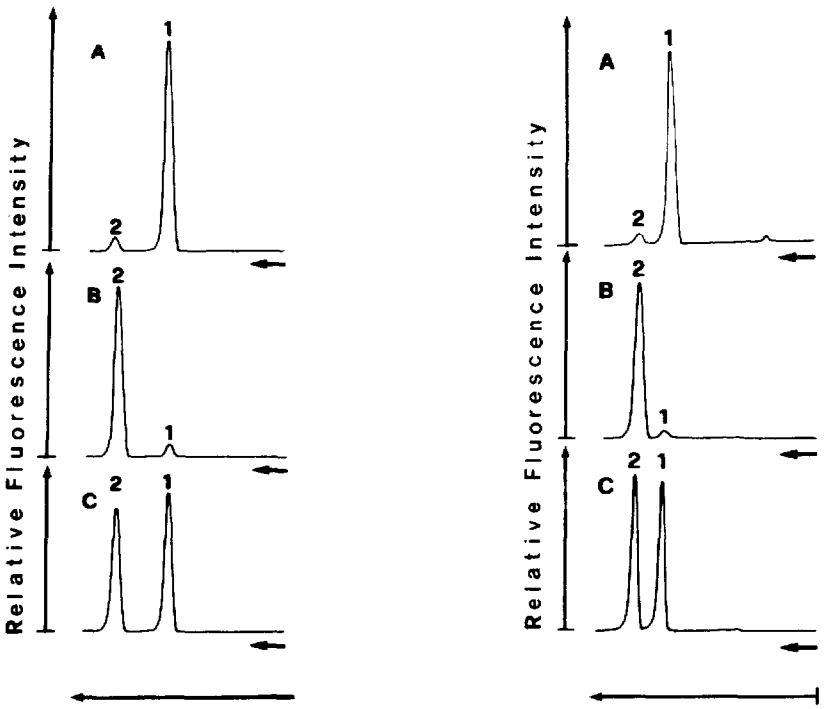

Fig. 3. Chromatograms of the HPLC determination (silica gel column; mobile phase cyclohexane—dichloromethane—tetrahydrofuran, 5:1:1, v/v) of α-methylbenzylamine (α-MBA) after extraction from plasma (5 μg/ml α-MBA) and derivatization with (*S*)-(+)-benoxaprofen chloride (1 = amide of (*R*)-(+)-α-MBA; 2 = amide of (*S*)-(—)-α-MBA). (A) (*R*)-(+)-α-MBA; (B) (*S*)-(—)-α-MBA; (C) racemic α-MBA.

Fig. 4. Chromatograms of the HPLC determination of tranylcypromine (TCP) after extraction from plasma (5 μg/ml TCP) and derivatization with (*S*)-(+)-benoxaprofen chloride (1 = amide of (*S*)-(—)-TCP; 2 = amide of (*R*)-(+)-TCP). (A) (*S*)-(—)-TCP; (B) (*R*)-(+)-TCP; (C) racemic TCP.

The chromatographic (TLC and HPLC) behaviour of several optically active amines after extraction from plasma and derivatization with (*S*)-(+)-benoxaprofen chloride is shown in Table I. Figs. 1—5 show chromatograms of different amines after extraction, derivatization and TLC or HPLC separation. It is evident that the resulting diastereoisomers differ in their R_F values, or retention times, or both. In Fig. 6 are shown calibration curves of (*R*)-(+)-tranylcypromine derivatized with (*S*)-(+)-benoxaprofen chloride and (*R*)-(+)-tranylcypromine derivatized with (*R*)-(—)-benoxaprofen chloride.

Apart from their chemical stability and intense fluorescence, the configurational stability of the (*R*)-(—)- and (*S*)-(+)-benoxaprofen chlorides is a marked advantage. (+)-Benoxaprofen and (—)-benoxaprofen can be stored at room temperature for at least twelve months without racemization.

In drug therapy numerous substances are administered as racemates. Often not only pharmacodynamics but also pharmacokinetics of the two optical isomers are different. Therefore it is essential to have analytical methods at one's disposal by means of which both enantiomers can be assayed separately in biological material. This can be accomplished in one analytical procedure

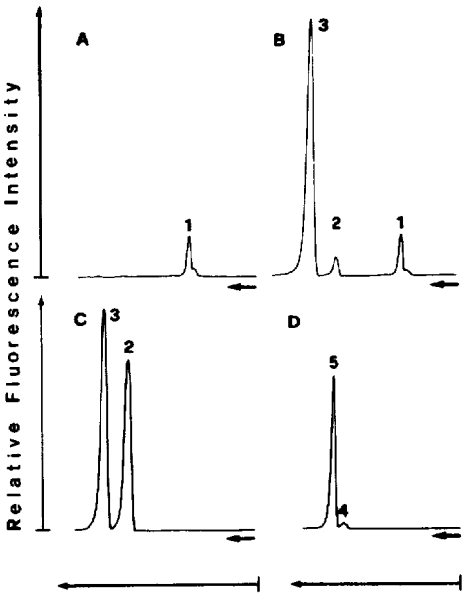

Fig. 5. HPLC determination of amphetamine and methamphetamine after extraction from plasma (5 μg/ml amine) and derivatization with (*S*)-(+)-benoxaprofen chloride (1 = benoxaprofen, 2 = amide of (*R*)-(—)-amphetamine, 3 = amide of (*S*)-(+)-amphetamine, 4 = amide of (*R*)-(—)-methamphetamine, 5 = amide of (*S*)-(+)-methamphetamine). (A) Blank plasma; (B) (*S*)-(+)-amphetamine; (C) racemic amphetamine; (D) (*S*)-(+)-methamphetamine.

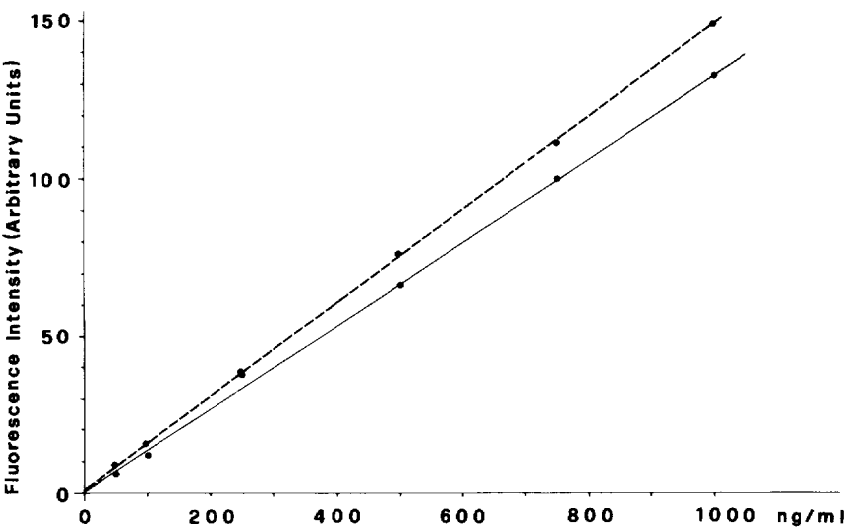

Fig. 6. Calibration curves of (*R*)-(+)-tranylcypromine extracted from different plasma standards and derivatized with (*S*)-(+)-benoxaprofen chloride (•——•) or (*R*)-(—)-benoxaprofen chloride (•- - -•) (TLC determination).

using our method. Because of insufficient or very complicated analytical methods the simultaneous determination of plasma levels and kinetic parameters of both enantiomers has up to now only been performed for a few optically active drugs. In particular, information is lacking on the extent to

which the optical isomers affect each other in their kinetics, and whether the transformation of one isomer into the other in the organism, which has been observed with some substances [17, 18], also occurs in other groups of drugs.

With activated benoxaprofen it is possible to derivatize not only substrates with amino groups but also those with hydroxyl groups [13]. Therefore this reagent can also be applied to the determination of optically active compounds with hydroxyl groups. Many racemic drugs contain either amino or hydroxyl groups or both. Thus, also β-adrenoceptor blocking agents (e.g. propranolol, metoprolol, atenolol) can be derivatized using activated benoxaprofen for their simultaneous stereoselective determination [19].

The described optically active fluorescent labels enable the quantitative determination of optically active amines and alcohols even at low concentrations [13] and in biological material. This is a marked advance in the development of efficient analytical assay methods for pharmacokinetic studies.

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