Journal of Chromatography, 427 (1988) 131–137
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4119

Note

S-(+)-Flunoxaprofen chloride as chiral fluorescent reagent

H. SPAHN*

Pharmakologisches Institut für Naturwissenschaftler der Johann Wolfgang Goethe-Universität, Theodor-Stern-Kai 7, Gebäude 75 A, D-6000 Frankfurt/M. 70 (F.R.G.)

(First received October 16th, 1987; revised manuscript received January 6th, 1988)

For the enantiospecific analysis of drugs and drug metabolites in biological fluids and tissues, highly efficient and sensitive assay methods are essential. Although direct resolution is certainly preferable, if it works, procedures based on the formation of diastereomeric derivatives by covalently binding the substrates are mostly used, and among these procedures by means of which small amounts of the xenobiotics can be detected.

In 1984 the acyl chloride of the intensely fluorescent benoxaprofen [BOP; 2-(*p*-chlorophenyl)-α-benzoxazoleacetic acid], a non-steroidal anti-inflammatory compound from the group of 2-arylpropionic acids, was described as a chiral acylating agent for optically active amines, forming highly fluorescent diastereomeric products [1]. However, before a BOP derivative can be used as a chiral marker, semipreparative resolution of the racemate into its enantiomers is necessary, an inevitable but rather time-consuming step for the preparation of the reagent. In this subgroup of non-steroidal anti-inflammatory drugs (NSAIDs) the dextrorotatory enantiomer is the eutomer, i.e., it is usually more active than the levorotatory enantiomer. The IC_{50} values were found to be significantly higher for the *R*-enantiomer in prostaglandin synthetase models.

Naproxen [NAP, *S*-(+)-6-methoxy-α-methyl-2-naphthalineacetic acid], another compound from the group of 2-arylpropionic acids, is marketed as the enantiomerically highly pure *S*-(+)-enantiomer. NAP exhibits a lower, but still remarkable fluorescence and is therefore also suitable as a chiral marker after activation to its acyl chloride [2,3], with the advantage of being available as the pure *S*-enantiomer. Naproxen chloride (NAP-Cl) can be used for chiral deriva-

*Present address: School of Pharmacy, Department of Pharmacy, University of California, San Francisco, CA 94143-0446, U.S.A.

0378-4347/88/$03.50

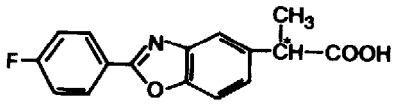

Fig. 1. Structure of flunoxaprofen

tization, if the concentrations of the compounds to be determined are in a range that does not require a strongly fluorescent marker, or if these substrates possess their own chromophores. Recently, another optically active benzoxazole derivative has been described in the literature (e.g. ref. 4), which, as far as the chemical structure is concerned, is closely related to BOP: flunoxaprofen (FLOP) (Fig. 1).

FLOP [*S*-(+)-(*p*-fluorophenyl)-α-methyl-5-benzoxazoleacetic acid] is also used as non-steroidal anti-inflammatory agent. As in the case of NAP it is the *S*-enantiomer that is therapeutically used.

In this communication the applicability of flunoxaprofen chloride (FLOP-Cl) as a chiral derivatization reagent and as possible alternative to benoxaprofen chloride (BOP-Cl) is described. α-Methylbenzylamine (α-MBA) and tranylcypromine (TCP) enantiomers were used as substrates for FLOP-Cl, and a resolution of their diastereomeric derivatives demonstrated by high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC).

EXPERIMENTAL

Chemicals

S-, *R*- and *R/S*-flunoxaprofen were kindly provided by Ravizza (Muggio, Italy). *S*-Benoxaprofen was prepared according to Weber et al. [1]. Tranylcypromine enantiomers and racemate were donated by Röhm Pharma (Weiterstadt, F.R.G.). The enantiomeric purities were: 1*S*,2*R*-(+)-*trans*-phenylcyclopropylamine [(+)-TCP] = 97.8% and 1*R*,2*S*-(−)-*trans*-phenylcyclopropylamine [(−)-TCP] = 98.1%, i.e., $e = 0.956$ (+) and 0.962 (−) ("*e*" is defined as a measure for the excess of one enantiomer with respect to the other one). α-Methylbenzylamine racemate and enantiomers were purchased from EGA (Steinheim, F.R.G.). Ethylchloroformate, triethylamine and L-leucinamide hydrochloride were from Fluka (Buchs, Switzerland). Silica gel TLC plates without fluorescent indicator, as well as the solvents, which were analytical grade, and the LichroCart 4-4 guard cartridges were purchased from E. Merck (Darmstadt, F.R.G.).

Equipment

The HPLC system consisted of a Knauer HPLC pump 64 (Knauer, Berlin, F.R.G.), a Rheodyne injection valve with a 10-μl sample loop (Rheodyne, Cotati, CA, U.S.A.), a Zorbax Sil HPLC column (25 cm × 0.46 cm I.D., 7 μm particle size, DuPont, Wilmington, DE, U.S.A.), a Shimadzu RF 530 fluorescence monitor (Shimadzu, Kyoto, Japan) and a Knauer recorder.

For TLC analyses solutions were applied on the plates using a Linomat III (Camag, Muttenz, Switzerland). The plates were scanned with a chromatogram spectrophotometer KM3 (Carl Zeiss, Oberkochen, F.R.G.) after chromatography.

Estimation of the enantiomeric purity of S-(+)-flunoxaprofen

For this purpose the amino acid derivative L-leucinamide [5,6] was used as the coupling component. To 1 μg of racemic flunoxaprofen or *S*-(+)-flunoxaprofen, respectively, 100 μl of a solution of triethylamine in dried acetonitrile (50 m*M*) were added, and the tube was vortexed briefly. Then 50 μl of a solution of ethylchloroformate in dried acetonitrile (60 m*M*) and after 2 min 50 μl of a solution of L-leucinamide hydrochloride (1 *M*) and triethylamine (1 *M*) in methanol were added. After an additional 3 min the reaction was stopped with 0.2 ml of 0.25 *M* hydrochloric acid. The products were extracted with 5 ml of ethyl acetate. After evaporation of the solvent, the residue was dissolved in 200 μl of mobile phase (given below), and 3 μl were injected. The chromatographic conditions were as follows: Zorbax Sil column (25 cm × 0.46 cm I.D., 7 μm particle size) and a silica gel guard column LichroCart 4-4, filled with LiChrosorb Si 60; mobile phase, dichloromethane–methanol (50:1, v/v); flow-rate, 1 ml/min; temperature, ambient; medium pressure, 12.5 MPa. (All glassware had to be silanized including glass vials of automatic sample injection systems).

S-Benoxaprofen with a known enantiomeric purity was analysed in the same way. Furthermore, the enantiomeric purity of FLOP was calculated based on the derivatization with TCP enantiomers with known enantiomeric purities (according to the derivatization and separation procedure given below, HPLC data).

Preparation of the FLOP-Cl reagent solution

To 1 mg of flunoxaprofen enantiomer, 0.5 ml of thionyl chloride (freshly distilled over linseed oil) were added. The mixture was heated to 80°C for 1 h to prepare the acyl chloride. Then the excess of thionyl chloride was completely evaporated, and 10 ml of dichloromethane (dried over a 4-Å molecular sieve) were added. This solution was the reagent stock solution. A 1:10 dilution was prepared for the derivatization of amines in a concentration range that is close to the one occurring in biological materials.

FLOP-Cl was also synthesized in larger amounts as described previously for BOP-Cl [1]: 1 mmol of flunoxaprofen enantiomer was dissolved in 25 ml of toluene and 2.5 ml of thionyl chloride were added. The mixture was refluxed for 30 min and the solvents evaporated. The resulting residue was dried over potassium hydroxide in vacuum. Recrystallization was possible from dichloromethane. If stored under anhydrous conditions, the resulting compound is stable for at least two months [m.p. 73°C (obtained with a Büchi apparatus), IR (cm^{-1}): 1780 (>C=O, acyl chloride)].

Derivatization of optically active amines

To 1 μg of amine, 0.2 ml of the reagent solution (1:10 dilution) were added. The tube was allowed to stand at room temperature for 60 min. Then the solvent was evaporated and the residue reconstituted in 100 μl of dichloromethane. (For substrates other than α-MBA and TCP, addition of a base can increase the yield.)

The derivatization was performed in a similar way after extraction of the amines from biological material. In the case of tranylcypromine the procedure for plasma and urine was as follows: To 1 ml of plasma (blank or 1 μg TCP per ml), 1 ml of pH 11 buffer (borate, sodium hydroxide) was added. The aqueous layer was ex-

tracted with 2.5 ml of diisopropyl ether–ethanol (10:0.15, v/v), and 2 ml of the organic layer were transferred into another tube and evaporated at ambient temperature using a vacuum centrifuge. Urine samples were treated in the same way, but using 0.05 *M* sodium hydroxide instead of a buffer solution. To the resulting residue, 0.2 ml of reagent solution was added. The solution was allowed to stand at room temperature for 60 min, and the reaction was stopped by adding 20 μl of methanol. The solvents were evaporated and the residue was reconstituted in dichloromethane. The diastereomeric FLOP-TCP amides that were formed, as well as the amides of α-MBA, can be separated by HPLC or TLC.

HPLC conditions. A good resolution was obtained by HPLC on a silica gel column with cyclohexane–dichloromethane–tetrahydrofuran (7:1:1, v/v) as mobile phase with a flow-rate of 1.4 ml/min at ambient temperature (6 MPa as medium pressure). The detector was set at 305 nm for excitation and 355 nm for emission. The injection volume was 5–50 μl.

TLC conditions. Resolution by TLC was achieved on TLC plates precoated with silica gel 60 with toluene–tetrahydrofuran–dichloromethane (5:1:1, v/v) as mobile phase. The plates were developed in an unlined glass tank after a saturation time of 15 min. After being air-dried, the plates were scanned at 313 nm for excitation and 365 nm for emission (both monochromatic filters).

HPLC and TLC resolution

The HPLC resolution was characterized by calculating the capacity k', the separation factor α and the resolution factor R as described by e.g. Testa [8]. To characterize the TLC resolution, parameters were calculated based on the retention factors R_F (of peak 1 and peak 2) in a similar way ($\alpha = R_{F2}/R_{F1}$; $R = (R_{F2} - R_{F1})/0.5(w_1 + w_2)$, where w_1 and w_2 are the widths of peaks as R_F equivalents).

Detection limit, linearity and reproducibility

Blank plasma was spiked with different amounts of racemic TCP (200, 400, 600, 800 and 1000 ng/ml). The samples were treated as described above. The coefficient of variation (C.V.) was estimated by analysing twelve samples, each containing 1 μg/ml. The detection limit was estimated after extraction of tranylcypromine from water and from plasma standards.

RESULTS

L-Leucinamide has been shown to exhibit a high enantiomeric purity [6]. Using L-leucinamide as the coupling component, the enantiomeric purity of *S*-flunoxaprofen could therefore be estimated. The described HPLC conditions gave a very good resolution of the corresponding diastereomeric derivatives. The k' values were 4.52 for the *S*-flunoxaprofen and 8.63 for the *R*-flunoxaprofen derivative. The separation and resolution factors, α and R, were 1.91 and 11.24, respectively.

The enantiomeric purity e, calculated according to Bähr and Theobald [9], under the assumption of $e = 1$ for L-leucinamide, was 96.1% ($e = 0.922$) for *S*-

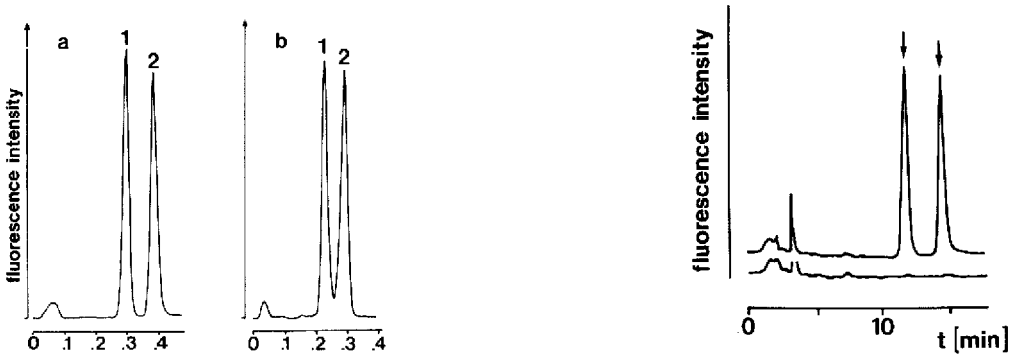

Fig. 2. TLC separation of diastereomeric derivatization products of α-MBA (a) and TCP (b) with *S*-FLOP-Cl. The *x*-axis gives the retention (R_F). Peaks: (a) 1 = *S*-(−); 2 = *R*-(+); (b) 1 = *R*-(+); 2 = *S*-(−).

Fig. 3. HPLC resolution of the diastereomeric amides resulting from derivatization of *R*/*S*-(±)-TCP with *S*-FLOP-Cl after extraction from plasma (containing 1 μg of racemic TCP per ml), compared with blank plasma. Elution order of the derivatives on a silica gel stationary phase: 1 = *S*-(−); 2 = *R*-(+).

flunoxaprofen. Based on the data from HPLC analysis of the FLOP derivatives of TCP enantiomers with known enantiomeric purity (see *Chemicals*), *e* was found to be 0.928. Using L-leucinamide, a similar chromatographic resolution as for the flunoxaprofen derivatives was found for the benoxaprofen derivatives. For BOP the corresponding k' values were 3.82 (*S*) and 7.52 (*R*), with $\alpha = 1.97$ and $R = 10.25$; the enantiomeric purity for *S*-BOP was 95.4% ($e = 0.908$), compared with 96.5% ($e = 0.930$) using a chiral column [7]. Impurity data obtained from TLC were generally higher (factor 1.5), indicating configurational changes of the investigated amides on the TLC plate in the presence of ammonia.

The fluorescence intensity of flunoxaprofen appeared to be in the same range as that of benoxaprofen, and the detection limits of the compounds were similar at their excitation and emission maxima (λ_{max} (FLOP) for TLC, excitation = 305 nm, emission = 350 nm; for HPLC, excitation = 305 nm, emission = 355 (365) nm). he molar extinction coefficient was determined to be 20% lower for flunoxaprofen than for benoxaprofen (30 000) in methanol.

Generally, the diastereomeric products of optically active amines with FLOP-Cl are easily separable by TLC and HPLC. Being more hydrophilic than the BOP-Cl products, they exhibit shorter retention times on reversed-phase and longer retention times on silica gel stationary phases. The retention on silica TLC plates and the resolution of the diastereoisomers formed from the test substrates, which are α-methylbenzylamine and tranylcypromine are shown in Fig. 2 and Table I. Fig. 3 depicts the HPLC resolution of the diastereomeric amides formed from racemic TCP.

In the case of TCP, spiked plasma samples were also extracted as described

TABLE I

RESOLUTION OF *S*-FLOP DERIVATIVES BY SILICA GEL TLC AND BY NORMAL-PHASE HPLC

k' values characterize the relative retention for HPLC; R_F values characterize the retention of the compound on the TLC plate in relation to the mobile phase front; α-MBA = α-methylbenzylamine; TCP = tranylcypromine.

Derivatives of	TLC				HPLC			
	R_{F1}	R_{F2}	α	R	k'_1	k'_2	α	R
R/S-α-MBA	0.306	0.403	1.32	2.10	2.63	5.39	2.05	6.37
R/S-TCP	0.240	0.310	1.29	1.13	4.33	5.63	1.30	2.68

previously for TCP (using BOP-Cl for derivatization [1]). At a TCP concentration of 1 μg/ml (racemic drug) in plasma and urine, no interfering peaks from plasma or urine constituents were found, whereas the TCP enantiomers were clearly detectable. A linear correlation between the peak areas and the concentrations was found within the investigated concentration range (200 ng/ml to 1 μg/ml) with coefficients of correlation of 0.995 for the *R*-enantiomer and 0.993 for the *S*-enantiomer. The intra-day coefficient of variation at a concentration of 1 μg racemic TCP per ml plasma was 8.1%. The detection limit (for each peak) for TCP was 1 ng/ml, when extracted from water, and 2 ng/ml in plasma.

DISCUSSION

The results from the present investigations with *S*-flunoxaprofen chloride demonstrate that this compound is suitable for chiral derivatization of optically active amines. The separation and resolution factors of the derivatives are in a similar range as those of the diastereomeric derivatives formed with benoxaprofen chloride and sufficient for the development of assay methods. HPLC in particular gave well resolved, almost symmetric peaks with reasonable k' values. Extraction of e.g. TCP from plasma and urine prior to derivatization is possible in the same way, as previously shown for the derivatization of TCP with BOP-Cl [1].

Activation to the acyl chloride is easy to perform, the conversion of FLOP into FLOP-Cl being almost complete. The limit of detection for the highly fluorescent FLOP is in the lower picomole range.

Owing to the high reactivity of the acyl chloride (towards alcohols and amines) it has to be ensured that interfering compounds can be well separated from the diastereomeric derivatives, either by extraction or by a suitable chromatographic procedure. An intrinsic property of all chiral fluorescent reagents up to now (e.g. naphthylethyl isocyanate [10] and including flunoxaprofen chloride) is the fact that decomposition products and by-products usually possess the same chromophores as the desired products themselves, and there is no derivatization that selectively occurs for the chiral amine.

On the other hand, the advantages of the highly fluorescent FLOP can be summarized as follows: FLOP is available as the *S*-enantiomer, stable in its non-activated form, and can easily be activated using e.g. thionyl chloride, forming the derivatization reagent (acyl chloride of FLOP) which, being a solid compound, is easier to handle than the volatile and lachrymatory isocyanates (phenylethyl isocyanate: b.p. 55 °C). *S*-FLOP has already been successfully applied to the stereospecific assay of baclofen after p.o. and i.v. administration in rats and humans [11].

ACKNOWLEDGEMENTS

The author thanks Mrs. Gabriele Hahn for technical assistance. The help of Mrs. Dorit Krauss and the support of Prof. Dr. Dr. E. Mutschler (Department of Pharmacology, University of Frankfurt/M.) are also appreciated, as well as the support of Ravizza S.p.A., especially Dr. A. Forgione, who kindly provided flunoxaprofen racemate and enantiomers.

REFERENCES

1 H. Weber, H. Spahn, E. Mutschler and W. Mőhrke, J. Chromatogr., 307 (1984) 145.
2 H. Spahn and W. Henke, Naunyn-Schmiedeberg's Arch. Pharmacol., 329 (Suppl.) (1985) R11.
3 H. Spahn, D. Krauss and E. Mutschler, Pharm. Res., 5 (1988) 107.
4 F. Berti, G. Galli, G. Rossini, G. Brunelli, L. Daffonchio, T. Vigano, F. Magni, M.T. Crivellari, A. Forgione and G.C. Folco, Drug Res., 37 (1987) 27.
5 S. Bjőrkman, J. Chromatogr., 339 (1985) 339.
6 K.H. Lehr and D. Damm, ISSX 2nd European Symposium on Foreign Compound Metabolism, Frankfurt/M., March/April 1987.
7 G. Pflugmann, H. Spahn and E. Mutschler, J. Chromatogr., 416 (1987) 331.
8 B. Testa, Xenobiotica, 16 (1986) 265.
9 W. Bähr and H. Theobald. Organische Stereochemie, Springer Verlag, Berlin, Heidelberg, New York, 1973 p. 71.
10 G. Gübitz and S. Mihellyes, J. Chromatogr., 314 (1984) 462.
11 D. Krauss, Ph.D. Thesis, Frankfurt/M., 1988.