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Fluorescence assay for small peptides and amino acids: high-performance liquid chromatographic determination of selected substrates using activated *S*-flunoxaprofen as a chiral derivatizing agent

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SUMMARY

The applicability of the fluorescent *S*-flunoxaprofen, activated to the corresponding acyl chloride, as a chiral derivatizing agent for amino acids and small peptides was evaluated. The amino acid or peptide solution was evaporated to dryness. The carboxylic moieties were esterified with 3 *M* hydrochloric acid in 2-propanol. The solvent and hydrochloric acid were evaporated, and the residue was dissolved in aqueous sodium heptanesulphonate and extracted with benzene–butanol (85:15, v/v). The organic phase was evaporated and *S*-flunoxaprofen chloride reagent solution (in dichloromethane) added, together with 20 mg of anhydrous sodium carbonate. After 60 min at 40°C the solvent was evaporated and the residue reconstituted in mobile phase (*n*-hexane–dichloromethane–ethanol). Resolution of the diastereomeric derivatives was accomplished on a Zorbax Sil high-performance liquid chromatography column. The eluate was monitored either fluorimetrically at 305/355 nm or by measuring the UV absorption at 305 nm. The procedure leads

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to highly fluorescent derivatives of amino acids or small peptides, which are resolvable on silica gel stationary phases.

INTRODUCTION

Peptide xenobiotics are receiving increased attention and, consequently, procedures for their specific assay in quality control, drug release assessment and pharmacokinetics are gaining importance as well. Highly potent peptide drugs may be dosed very low, resulting in low concentrations in the effect compartment or in the accessible blood compartment. Monitoring the enantiomeric composition may be of particular importance during protein or peptide synthesis. Trace analysis of impurities (closely related peptides or peptide fragments with potentially different effects), of stereoisomers and of degradation products (again including stereochemical aspects) may also be a crucial problem in pharmaceutical preparations [1].

A simple approach is the use of chiral columns, where the chiral discriminator is attached to the stationary phase, or the use of chiral eluents. However, the detection of trace amounts of amino acids can create problems. In order to increase the detectability of such a small peptide drug, a peptide drug isomer or a degradation product, fluorescence labelling may be employed.

Several fluorescence labelling agents are known for proteins, peptides and amino acids, including *o*-phthaldialdehyde (OPA) and fluorescamine, both of which react with primary amines only [2]. Another well known label is fluorescein, which is used as the isothiocyanate for primary and secondary amino group derivatization. None of these procedures permits the stereospecific assay of amino acids in an achiral chromatographic system.

Recent approaches to the enantio- or stereospecific detection of protein elements have also involved chiral derivatization reagents: e.g. amino acid derivatives (Boc-L-Leu-hydroxysuccinimide ester [3], N-acetylcysteine- or penicillamine–OPA derivatives [4]) and carbohydrate derivatives (e.g. 2,3,4,6-tetra-O-acetyl-1-thio-β-glucopyranoside [5], derivatives formed with OPA and the sodium salt of 1-thio-β-D-glucose [6] or related carbohydrates [7]). Amino acids and carbohydrates themselves are poorly detectable in small amounts, unless they are coupled to a chromophor, such as the isoindole ring of OPA derivatives. The reagents that are based on OPA, although yielding highly fluorescent diastereomeric products, are not suitable for the derivatization of secondary amines.

2-Arylpropionic acid derivatives, which were first introduced as fluorescent reagents by our group in 1984 [8], and fluorenylethylchloroformate [9] represent alternatives, since they are chiral and fluorophoric labels. *S*-(−)-N-1-(2-Naphthylsulphonyl)-2-pyrrolidinecarbonyl chloride (NSP-Cl) was applied for the assay of amino acid enantiomers with measurement of the UV

absorbance [10]. Activated 2-arylpropionic acids had previously been used by our group to assay drugs that are structurally related to amino acids (baclofen and analogues) [11].

A strongly chiral fluorescent marker with excitation and emission wavelengths distinct from those of most endogenous compounds is the 2-arylpropionic acid flunoxaprofen, which was recently introduced as a chiral coupling component for amines when activated to its acyl chloride (*S*-(+)-flunoxaprofen [12]) or isocyanate (*S*-(−)-flunoxaprofen isocyanate [13]). Hence it appeared reasonable to apply *S*-flunoxaprofen to trace analysis of amino acids and small peptides as well. The derivatization steps for the reaction with an acyl chloride are shown in Fig. 1.

The objectives of the present studies were to develop an analytical procedure for amino acids and small peptides and to find out whether this procedure is also suitable for the identification of the amino acid composition of smaller peptides, such as those that may occur as decomposition products of larger peptides. As a reagent for primary and secondary amino groups it should also be suitable for N-methylated amino acids, which may be elements of drug peptide chains because of their stabilizing effect towards enzymic hydrolysis.

EXPERIMENTAL

Materials

2-Propanol, dichloromethane, *n*-hexane, benzene, *n*-butanol, ethanol, anhydrous sodium carbonate (all analytical grade) and thionyl chloride were from

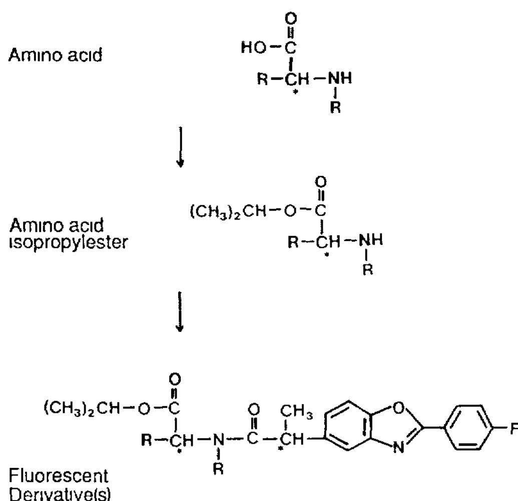

Fig. 1. Derivatization scheme for amino acids with 2-propanol–hydrochloric acid and flunoxaprofen chloride *S*-Flunoxaprofen chloride was usually used as chiral derivatizing agent.

E. Merck (Darmstadt, F.R.G.), and sodium decyl hydrogensulphate was from Fluka (Buchs, Switzerland). They were used as received with the exception of thionyl chloride, which was freshly distilled over linseed oil before use. All amino acids, dipeptides and sodium azide were purchased from Sigma (Deisenhofen, F.R.G.). Baclofen was from Ciba-Geigy (Wehr, F.R.G.). *S*-, *R*- and *R*/*S*-flunoxaprofen were kindly provided by Ravizza (Muggio, Italy).

Metkephamid was a gift from Eli Lilly (Indianapolis, IN, U.S.A.).

Apparatus

For the HPLC separations the following instruments were used: a Waters Model 600 multi-solvent delivery system, a Waters WISP 712 intelligent sample processor, a Waters Model 481 LC spectrophotometer (Waters, Milford, MA, U.S.A.), a Knauer Model 64 HPLC pump (Knauer, Berlin, F.R.G.), a Shimadzu RF-530 fluorescence monitor and a Shimadzu C-R5A Chromatopac integrator (Shimadzu, Kyoto, Japan) and an automatic solvent degasser (Erma, Tokyo, Japan).

The HPLC silica gel column (Zorbax-Sil, 5 μm, 250 mm$\times$4.6 mm I.D.) was purchased from DuPont (Wilmington, DE, U.S.A.).

HPLC conditions

The mobile phases were different combinations of *n*-hexane–dichloromethane–ethanol (I, 100:10:1.5, v/v; II, 100:10:0.8, v/v) and *n*-hexane–chloroform–ethanol (III, 100:10:1, v/v). The flow-rate was 2.0 ml/min at ambient temperature with a back-pressure of 4.2 MPa (600 p.s.i.) for I, 8.5 MPa for II and 6.1 MPa for III. For dipeptide derivatives a fourth mobile phase was used: dichloromethane–*n*-hexane–*n*-propanol (150:70:6.5, v/v) at 1.5 ml/min and 9.6 MPa. Detection was by UV absorption at 305 nm and/or fluorescence measurement (305 nm excitation, 355 nm emission).

Preparation of the reagents

S-Flunoxaprofen chloride was prepared by treating the carboxylic acid with thionyl chloride (in toluene) as reported earlier [12] with the addition of trace amounts of dimethylformamide.

For the screening and optimization of mobile phases *R*/*S*-flunoxaprofen chloride can also be used for derivatization, since the two pairs of diastereomers that result from an amino acid racemate and either *R*- or *S*-flunoxaprofen chloride have identical retention behaviour on an achiral stationary phase. When only one of the amino acid enantiomers was available, as was the case with N-methylated amino acids, the retention behaviour of the derivative of the second enantiomer was simulated by derivatization with *R*- instead of *S*-flunoxaprofen chloride.

The chiral derivatizing agent was dissolved in anhydrous dichloromethane or ethyl acetate at a concentration of 1 mg/ml.

For comparison of the chromatographic properties of the products, small amounts of the corresponding isocyanate were prepared as well, according to the procedure that was recently described by our group [13]. Derivatization of amino acid esters was performed as described below.

Derivatization of amino acids and peptides with flunoxaprofen chloride

The carboxylic moieties of the amino acids and peptides were esterified by treating them with 3 *M* hydrochloric acid in 2-propanol at 105°C for 20 min. Afterwards the liquid was evaporated under nitrogen at the same temperature. The residue was dissolved in 1 ml of aqueous sodium heptanesulphonate (5 mg/ml) and extracted with 5 ml of benzene–butanol (85:15, v/v) or with dichloromethane. The organic layer was evaporated at 80°C for benzene–butanol or 50°C for dichloromethane. *S*-Flunoxaprofen chloride solution was then added, yielding an at least three-fold molar excess, together with 20 mg of anhydrous sodium carbonate. After 60 min at 40°C, or moderate agitation at ambient temperature overnight, the solvent was evaporated and the residue reconstituted in 200 μl of mobile phase, and the supernatant pipetted into separate vials. The injection volume was 10–20 μl.

Comparison of the chromatographic behaviour of amides and urea derivatives

From phenylalanine or leucine ethyl and isopropyl esters, derivatization was performed with flunoxaprofen chloride (as given above) and, in addition, with the corresponding isocyanate (as described in ref. 13), yielding diastereomeric amides or ureas. Chromatographic conditions were similar for both derivatization procedures: Zorbax Sil as stationary phase and mobile phase I.

Hydrolysis conditions for peptides

Acid treatment. To ≤5 mg of peptide, 200 μl of 6 *M* hydrochloric acid were added in a screw-capped tube. The tube was heated to100°C for 6 h, then the hydrochloric acid was evaporated at 80–100°C under a stream of nitrogen.

Alkali treatment. To ≤5 mg of peptide, 200 μl of 2 *M* sodium hydroxide solution were added in a screw-capped tube. After incubation at 100°C for 2 h, 100 μl of 5 *M* hydrochloric acid were added to neutralize the sodium hydroxide. The liquid was evaporated at 80–100°C under a stream of nitrogen.

After acid treatment, the flunoxaprofen chloride derivatization can be performed directly, i.e. without extraction. This is in principle possible for the alkaline hydrolysate as well, although the large amount of resulting inorganic salts will reduce the derivatization yield. In both cases, however, extraction prior to derivatization increases the reaction yield when the chiral derivatization is performed.

Correction of the enantiomer concentrations in hydrolysates

Owing to the enantiomeric impurity in the reagent, the results had to be corrected arithmetically according to the procedure of Hermansson and Von Bahr [14].

Detection limit

The detection limit was estimated by derivatization of decreasing amounts of amino acids. Three different types of amino acid were used: one with a short (leucine), one with a medium (methionine) and one with a long retention time (glycine). The detection limit was determined on the basis of isocratic elution and was thus expected to be dependent on the retention on the column.

Assay reproducibility

These studies were all performed with fluorescence detection because of the lower baseline noise and increased sensitivity compared with UV detection. Different amounts of alanine (0.1–2 μg) were derivatized with hydrochloric acid–2-propanol and flunoxaprofen chloride. The coefficient of variation (C.V.) was estimated by analysing two sets of twelve samples each containing 1 μg of L-alanine, one set without and one set with acid treatment prior to esterification and chiral derivatization.

Hydrolysis of model peptides for elucidation of their amino acid composition

The peptides that were hydrolysed via acid treatment were D-alanine-glycine, L-alanine-L-leucine, L-alanine-L-methionine, DL-alanine-DL-phenylalanine, DL-alanine-DL-leucine and metkephamid.

L-Alanine-L-leucine and DL-alanine-DL-leucine were furthermore hydrolysed via alkali treatment.

RESULTS AND CONCLUSIONS

Derivatization reactions

The derivatization reaction was found to be easy to perform and rapidly completed as expected for the derivatization of a (not sterically hindered) amine and an acyl chloride. An almost similar reactivity was found for the isocyanate. With the acyl chloride, the velocity of the derivatization reaction appeared slightly higher for the lipophilic amino acids, yet under the desired conditions the maximum yield was obtained for all substrates.

Chromatographic properties of the derivatization products

Prior to further detailed investigations, the resolution of the derivatization products of phenylalanine and leucine esters was investigated in the usually applied normal-phase chromatographic system. While the amides were always resolved into two distinct peaks, only one peak was obtained for the diaste-

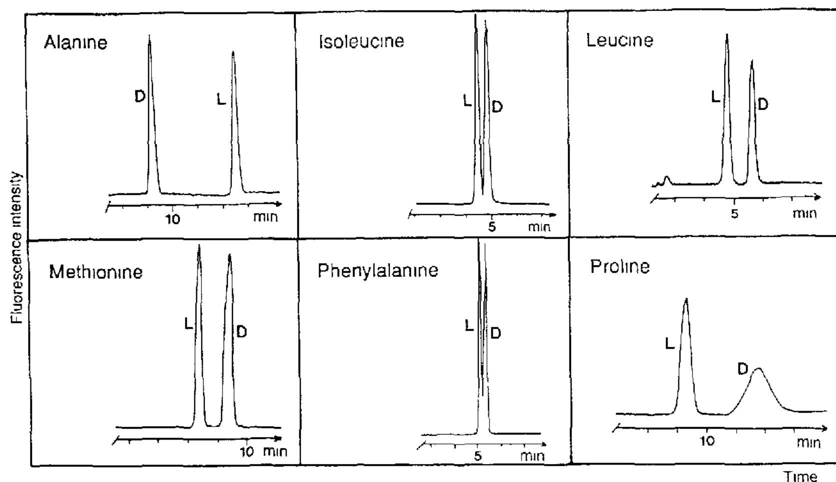

Fig. 2. HPLC behaviour of the S-flunoxaprofen chloride derivatives of several amino acid isopropyl esters on a Zorbax Sil column with n-hexane–chloroform–ethanol (100 · 10 : 1, v/v) as mobile phase (flow-rate, 2.0 ml/min).

reomeric urea derivatives. Chromatograms resulting from the flunoxaprofen–2-propanol derivatives of several amino acids, e.g. with aliphatic side-chains such as alanine, leucine and isoleucine, the sulphur-containing methionine, phenylalanine with an aromatic side-chain and the secondary amine proline, are depicted in Fig. 2. Chromatographic parameters for various amino acids are summarized in Table I.

Interestingly, the elution order of the amino acid ester derivatives was dissimilar. For example, the L-enantiomer of leucine or isoleucine eluted before the D-enantiomer when S-flunoxaprofen chloride was used, but the order was reversed for alanine. Further detailed investigations on the influence of the alcohol component on the elution behaviour of amino acid flunoxaprofen and naproxen derivatives are currently under way.

Dipeptides were found to exhibit very long retention times in the chromatographic systems for amino acids and were hence chromatographed with a stronger eluent. For example, a retention time of 45 min was then obtained for the derivative of S-flunoxaprofen chloride and L-alanine-L-leucine on a silica column at a flow-rate of 1.5 ml/min.

Enantiomeric stability of the reagent and the substrates during the derivatization procedure

The enantiomeric excess for S-flunoxaprofen was determined to be 0.96. Additional peaks that appeared after esterification and derivatization with flunoxaprofen chloride could completely be explained by this value, i.e. no significant racemization during the analytical procedure of either reagent or substrate was detected.

TABLE I

HPLC PARAMETERS FOR THE DIASTEREOMERIC *S*-FLUNOXAPROFEN DERIVATIVES OF SEVERAL AMINO ACID ISOPROPYL ESTERS

The capacity factors were calculated as $(t_R - t_0)/t_0$, where t_0 and t_R are the elution times of an unretained peak and the derivative, respectively. The separation factors represent the ratios of the capacity factors of two peaks, i.e. k'_2/k'_1. The resolution factor for peaks 1 and 2, R, was calculated as $R = (t_{R_1} - t_{R_2})/0.5(w_1 + w_2)$, where t_{R_1} and t_{R_2} are the retention times of two diastereomeric derivatives and w_1 and w_2 are their peak widths at the baseline.

Amino acid	Capacity factor (k')		Separation (α)	Resolution (R)
	D	L		
Alanine	11 2	15.2	1.36	8.42
Glycine	19.5		–	–
Isoleucine	4.8	4.5	1.06	1 40
Leucine	7.1	6.0	1.19	4.07
Methionine	11.9	10.5	1 13	2.70
Phenylalanine	6.6	6 4	1.03	1.00
Proline	15.0	12.3	1.22	3.09
Tyrosine	22.6	13.1	1.73	3.14

Detection limit and reproducibility

A linear correlation between the peak areas and the amount of amino acid derivatized was found within the investigated range, with a correlation coefficient of 0.995. The intra-day C.V. at an amount of 1 μg of L-alanine was 8.1% when no internal standard was used. Using baclofen together with methionine or another amino acid with similar elution behaviour as the amino acid under investigation (e.g. isoleucine for leucine) usually improved the C.V. by ca. 1–2%.

The detection limit (for each peak) was in the range 0.1–0.5 ng, if the retention time did not exceed 30 min. It could be further improved by increasing the injection volume.

Hydrolysis of peptides

Under the conditions given in Experimental the hydrolysis was found to be complete or almost complete. From the chromatograms in Fig. 3 it is obvious that acid hydrolysis occurs with configurational stability, whereas alkaline hydrolysis leads to partial racemization even with comparatively short incubation times.

Amino acid detection in peptide hydrolysates

As mentioned in the Introduction, a major aim of this study was the detection of amino acids in hydrolysates, in order to elucidate the structure of trace

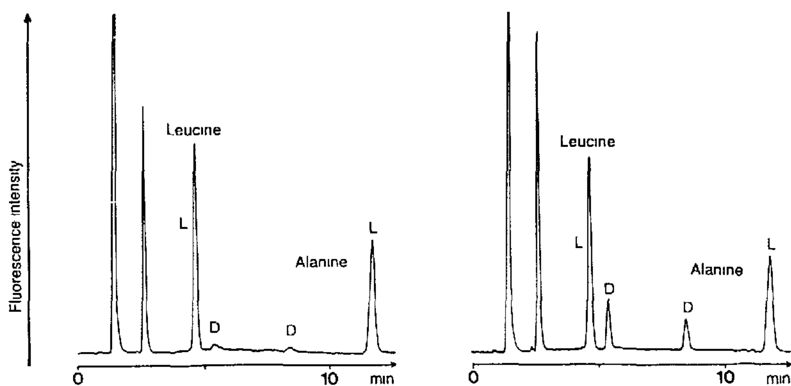

Fig. 3. Acid and alkaline hydrolysis of dipeptides (L-alanine-L-leucine as example).

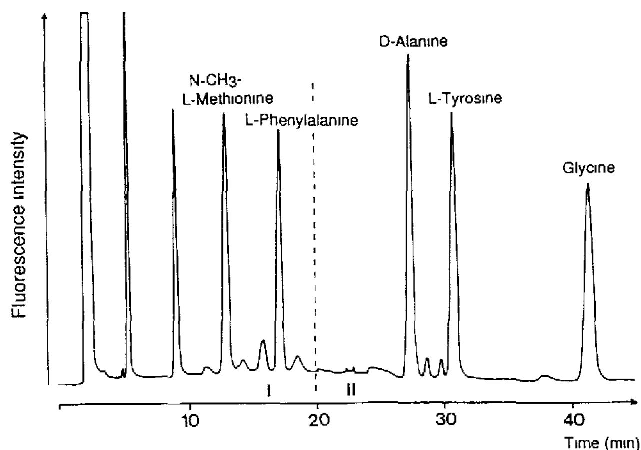

Fig. 4. Acid hydrolysis of metkephamid, yielding five amino acids. Chromatographic conditions: Zorbax Sil 5 μm; mobile phase I for 20 min, mobile phase II for 25 min, flow-rate, 2.0 ml/min; pressure 8.5 MPa; ambient temperature; detection wavelengths, 305/355 nm.

amounts of decomposition products of peptides. Hence, small peptides were hydrolysed to prove that the procedure is reliable. All investigated dipeptides were easily cleaved, the amino acids detected, and configuration was retained during hydrolysis. Hence two peaks were obtained for dipeptides composed of the L-enantiomers of the amino acids and four peaks for those composed of the D,L-mixtures of the amino acids. Hydrolysis of the pentapeptide metkephamid (L-tyrosine-D-alanine-glycine-L-phenylalanine-NCH_3-methionine-amide) yielded five peaks (Fig. 4). Four of these peaks were identified using the respective amino acid standards, but for the N-methylated methionine no reference compound was available. In spite of this, the peak that elutes next to

phenylalanine can be assigned to the methionine derivative, since it can be assumed that the more lipophilic derivative elutes earlier than methionine itself.

CONCLUSIONS

Recent studies on the use of the less fluorescent naproxen chloride as chiral discriminating agent for amino acid methyl esters [15,16] are in agreement with our findings. They were performed similarly to our HPLC procedure for baclofen enantiomers in biological material, but were followed by gas chromatographic or thin-layer chromatographic separation.

In summary, the proposed amino acid assay based on derivatization with fluorescent *S*-flunoxaprofen chloride is suitable for the trace analysis of various amino acid enantiomers. It further permits the determination of small peptides. Furthermore, preliminary studies indicate the applicability of the procedure for the elucidation of degradation products of peptides, including stereochemical aspects.

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REFERENCES

1 H.D. Jakubke and H. Jeschkeit, Aminosauren, Peptide, Protein, Verlag Chemie, Weinheim, 1982, p. 193.
2 D.R. Knapp, Handbook of Analytical Derivatization Reactions. Wiley, New York, 1979, p 68
3 A.R. Mitchell, S B.H. Kent, I.C. Chu and R.B. Merrifield, Anal. Chem., 50 (1978) 637
4 R.H. Buck and K. Krummen, J Chromatogr , 387 (1987) 255.
5 N. Nimura, A. Toyama and T. Kinoshita, J. Chromatogr., 316 (1984) 547.
6 S. Einarsson, S. Folestad and B Josefsson, J. Liq. Chromatogr., 10 (1987) 1589.
7 A. Jegorov, J. Triska, T. Trnka and M. Cerny, J Chromatogr., 434 (1988) 417.
8 H. Weber, H. Spahn, E. Mutschler and W. Möhrke, J. Chromatogr., 307 (1984) 167.
9 S. Einarsson, B. Josefsson, P. Möller and D. Sanchez, Anal. Chem., 59 (1987) 1191.
10 H. Nishi, K. Ishii, K Taku, R Shimizu and N. Tsumagari, Chromatographia, 27 (1984) 301.
11 H. Spahn, D. Krauss and E. Mutschler, Pharm. Res., 5 (188) 107
12 H. Spahn, J. Chromatogr., 427 (1988) 131.
13 E. Martin, K. Quinke, H Spahn and E. Mutschler, Chirality, 1 (1989) 223.
14 J. Hermansson and C. von Bahr, J. Chromatogr., 221 (1980) 109.
15 N. Büyüktimkin, S Büyuktimkin, D. Grunow and S. Elz, Chromatographia, 25 (1988) 925.
16 N. Büyüktimkin and A. Buschauer, J. Chromatogr., 450 (1988) 281.