*Journal of Chromatography*, 310 (1984) 167—178
*Biomedical Applications*
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2176

# α-ALKYL-α-ARYLACETIC ACID DERIVATIVES AS FLUORESCENCE MARKERS FOR THIN-LAYER CHROMATOGRAPHIC AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY OF AMINES AND ALCOHOLS

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(First received September 5th, 1983; revised manuscript received April 6th, 1984)

## SUMMARY

Activated *R,S*-benoxaprofen is described as a new reagent for fluorescent derivatization of drugs with primary or secondary amino groups or with hydroxyl groups. Separation of the reaction products is demonstrated by thin-layer chromatography and high-performance liquid chromatography. The sensitivity of the detection is in the picomole range. Derivatization procedures can be easily and rapidly performed.

## INTRODUCTION

The determination of drugs in biological materials usually requires very sensitive assay methods. Because of the low detection limit, the high specificity and the linearity in a large concentration range, measuring the fluorescence of the drugs has proved to be advantageous. Insufficiently fluorescent compounds with reactive groups can be converted to highly fluorescent derivatives by fluorescence markers [1]. The reagent should fulfil several requirements. First, it has to exhibit a high intensity of fluorescence; furthermore, it must react quantitatively and specifically with defined functional groups. The resulting products should be stable and possess chromatographic properties that make

0378-4347/84/$03.00 

the separation from the excess of the reagent and from by-products possible. In general, the fluorescence markers described in the literature and used up to now have several disadvantages; for example, instability, such as photoinstability, and formation of by-products or insufficient fluorescence yield [2].

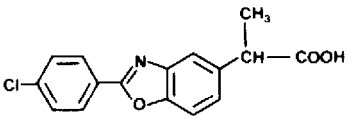


Fig. 1. Structural formula of benoxaprofen.

There are compounds in the chemical group of α-alkyl-α-arylacetic acids that are distinguished by pronounced absorption qualities or strong intrinsic fluorescence or both. For the investigations described in this paper, benoxaprofen [(*R*,*S*)-2-(*p*-chlorophenyl)-α-methyl-5-benzoxazoleacetic acid, Fig. 1), an antiinflammatory drug, which shows an intense fluorescence, was chosen from this group. Activation of benoxaprofen to a fluorescence marker (e.g. the corresponding acid chloride or imidazolide) can easily be performed. The agents obtained are characterized by a good reactivity leading to extraordinarily stable products when allowed to react with amines and alcohols.

## MATERIALS AND METHODS

### *Reagents and chemicals*

Solvents (analytical grade), thionyl chloride and thin-layer chromatography (TLC) plates (coated with silica gel 60 and reversed-phase material, RP-8 and RP-18) 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.). Carbonyldiimidazole was purchased from Serva (Heidelberg, F.R.G.), and α-methylbenzylamine from EGA (Steinheim, F.R.G.). Amphetamine, methamphetamine, β-phenylethylamine, benzylamine, methanol, ethanol, propanol, butanol, isoamyl alcohol, choline (all from Merck), maprotiline (Ciba-Geigy, Basel, Switzerland), tolylethylamine, phenylbutylamine (both from EGA), procaine (Hoechst, Frankfurt/M., F.R.G.) and scopolamine N-butylbromide (Boehringer Ingelheim, Ingelheim, F.R.G.) were also used as substrates for derivatization.

### *Apparatus*

Melting points were obtained with a Büchi apparatus and are uncorrected. Infrared (IR) spectra were recorded in potassium bromide discs with a Beckman Acculab 2 spectrophotometer.

Elemental analyses were performed by the Department of Organic Chemistry, University of Frankfurt (Prof. Dr. Ried) and analytical results are within 0.3% of the theoretical values.

TLC plates were scanned with a chromatogram-spectrophotometer KM3 (Carl Zeiss, Oberkochen, F.R.G.) and a recorder 56 (Perkin Elmer, Überlingen, F.R.G.).

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

HPLC was performed using a chromatograph LC 601 and a fluorescence detector 650-10 S (Perkin Elmer).

*Activation of benoxaprofen*

*Formation of the acid chloride.* Benoxaprofen (600 mg, about 2 mmol) was dissolved in 50 ml of dried toluene and, after slowly adding 5 ml of thionyl chloride (ca. a 20-fold amount, freshly distilled over linseed oil), the mixture was refluxed for 30 min. The solution was evaporated to dryness and the crystalline residue was recrystallized from dichloromethane if necessary.

*Formation of the imidazolide.* A solution of 500 mg of carbonyldiimidazole in 50 ml of dried dichloromethane was added to 100 mg of benoxaprofen. The solution was allowed to stand at room temperature for 30 min and was shaken occasionally. Then 5 ml of glacial acetic acid were added to destroy the excess of carbonyldiimidazole. The reagent solution obtained was used immediately.

*Characterization of the physical and spectral properties of benoxaprofen (BOP) and benoxaprofen chloride (BOP-Cl)*

m.p.: BOP = 191°C, BOP-Cl = 91.5°C. IR ($cm^{-1}$): BOP, 1700 (>C=O, acid); BOP-Cl, 1775 (>C=O, acid chloride).

Fluorescence spectra were scanned after chromatography on the TLC plate using the chromatogram-spectrophotometer KM 3. Excitation spectra: excitation, deuterium lamp; emission, monochromatic filter M 365. Emission spectra: excitation, mercury lamp (313 nm line).

HPLC fluorescence spectra were registered during chromatography (stop flow) using a fluorescence detector 650-10 S with a xenon lamp. Excitation spectra: emission wavelength = 365 nm. Emission spectra: excitation wavelength = 312 nm.

*Synthesis of reference compounds (derivatives of amines and alcohols)*

*α-Methylbenzylamine.* (Modified from Bopp et al. [3].) Benoxaprofen chloride (600 mg) was dissolved in 50 ml of dried dichloromethane. Then 10 ml of a solution of α-methylbenzylamine in dichloromethane (1.5 ml of α-methylbenzylamine and 8.5 ml of dichloromethane) were added slowly with stirring at room temperature. After refluxing for 3 h this 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: m.p.: 169°C, yield: 85%.

The solid gave one fluorescent spot when examined by TLC. Solvent system [3]: chloroform—methanol—water—ammonia (70:30:5:1, v/v). $R_F$ values: 0.89 (amide), 0.35 (BOP). IR ($cm^{-1}$): 1640 (> C=O, amide).

*Tranylcypromine.* The amide of tranylcypromine was synthesized with benoxaprofen chloride as described for the amide of α-methylbenzylamine. Because of the product's insolubility in dichloromethane it was easily isolated from the reaction mixture.

m.p.: 247°C, yield: ≈98%. TLC solvent system: cyclohexane—ethyl acetate—methanol (7:3:2, v/v), ammonia atmosphere, $R_F$ values: 0.43 (amide), 0.10 (BOP). IR ($cm^{-1}$): 1640 (>C=O, amide).

*Choline.* Benoxaprofen chloride (300 mg, about 1 mmol) and 420 mg of

choline chloride (about 4 mmol) in acetonitrile were refluxed for 1 h. The excess of choline chloride crystallized nearly quantitatively after cooling the reaction mixture. After centrifugation the solution was evaporated to dryness, and the residue was dissolved in water. This solution was shaken with toluene to remove benoxaprofen and evaporated to dryness again. The resulting ester was recrystallized from acetonitrile.

m.p.: 205°C, yield: ≈50%. TLC solvent system: acetone—methanol—formic acid (2:2:1, v/v), atmosphere of the glass tank saturated for 48 h. $R_F$ values: 0.36 (ester), 0.86 (BOP). IR ($cm^{-1}$): 1725 (>C=O, ester).

*Methanol.* Two drops of concentrated sulphuric acid were added to 200 mg of benoxaprofen chloride in 4 ml of methanol, and the mixture was heated to 80°C for 10 min. After cooling the ester crystallized from methanol. After centrifugation, methanol was decanted, the residue was washed with a mixture of water and methanol several times and then dried over phosphoric anhydride.

m.p.: 100.5°C, yield: ≈92%. TLC solvent system: toluene—dichloromethane—tetrahydrofuran (5:1:1, v/v). $R_F$ values: 0.78 (ester), 0.29 (BOP). IR ($cm^{-1}$): 1730 (>C=O, ester).

*Quantitative determination of primary and secondary amines*

*Reaction conditions for the derivatization with benoxaprofen chloride* (e.g. α-methylbenzylamine). To 10 μg of amine, 1 ml of a solution of benoxaprofen chloride (1 mg/ml) was added. The mixture was allowed to stand at room temperature. The investigation was performed with and without the addition of sodium and potassium carbonate (20 mg) and triethylamine (10 μl, freshly distilled) to the reaction mixture. The concentration of amide in the mixtures was assayed every 10 min after the reaction had been started. The TLC plates (silica gel, 5 × 10 cm) were developed immediately after application of the solutions using the synthesized amides as reference compounds.

*General procedure for the formation of derivatives with benoxaprofen chloride.* Up to 0.5 μmol of amine (or the residue after extraction from biological material) and 500 μl of benoxaprofen chloride in dried dichloromethane (1 mg/ml) were allowed to stand at room temperature for 60 min or heated to 50°C for 30 min.

*Formation of derivatives using the benoxaprofen imidazolide.* Up to 0.5 μmol of amine and 500 μl of a solution of benoxaprofen imidazolide (freshly prepared as described above) were allowed to stand at room temperature for 0.5 h.

*Extraction procedures from biological material.* Example 1: β-phenylethylamine from plasma. Plasma (1 ml), 1 ml of 0.1 *M* sodium hydroxide solution and 2.5 ml of *n*-hexane were mixed in a centrifuge tube. After shaking (20 min), the tubes were centrifuged briefly to separate the layers. Then 2 ml of the organic phase were transferred into another tube and evaporated to dryness using a vacuum centrifuge. The benoxaprofen chloride solution was added to the resulting residue. The derivatization was performed at room temperature.

Example 2: maprotiline from plasma. To 1 ml of plasma, 0.5 ml of potassium carbonate solution (10%) and 3 ml of *n*-hexane (freshly distilled) were added. After shaking (30 min) and centrifuging, 2 ml of the organic phase were transferred into another tube and evaporated to dryness using a vacuum

centrifuge. The benoxaprofen chloride solution was added. The mixture was heated to 50° C (30 min).

*TLC conditions.* Volume applied = 10 µl, band width = 5 mm. Solvent systems: (I) toluene—dichloromethane—tetrahydrofuran (5:1:1, v/v), ammonia atmosphere; (II) toluene—chloroform—tetrahydrofuran (5:4:1, v/v), ammonia atmosphere; (III) chloroform—methanol—formic acid—tetrahydrofuran (110:20:5:2, v/v); (IV) acetone—methanol—formic acid (2:2:1, v/v); (V) aqueous solution of sodium heptane sulphonate (0.01 mol/l)—acetonitrile—phosphoric acid (40:60:0.15, v/v); (VI) cyclohexane—ethyl acetate—methanol (7:3:2, v/v), ammonia atmosphere; (VII) methanol—water (9:1, v/v). Detection was by densitometric measurement of the intensity of fluorescence using a chromatogram-spectrophotometer KM3. 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.

*HPLC conditions.* Injection volume: 10 µl System a: analytical column (Dupont); 250 mm × 4.6 mm; stationary phase Zorbax-sil (7 µm) (Dupont); mobile phase cyclohexane-dichloromethane-tetrahydrofuran (5:1:1, v/v); ambient temperature; flow-rate 1 ml/min (at a pressure of 8.5 MPa). System b: analytical column (Knauer); 120 mm × 4.6 mm; stationary phase LiChrosorb RP-8 (5 µm) (Merck); mobile phase sodium heptane sulphonate (0.01 mol/l)—acetone—phosphoric acid (40:60:0.15, v/v); temperature 55° C; flow-rate 2 ml/min (at a pressure of 7.7 MPa). Detection was by fluorescence measurement; excitation wavelength 312 nm; emission wavelength 365 nm.

*Quantitative determination of alcohols*

*General procedure for the formation of derivatives with benoxaprofen chloride.* Up to 0.5 µmol of alcohol and 500 µl of a solution of benoxaprofen chloride (1 mg/ml) in acetonitrile, toluene or dichloromethane are heated to 60—80° C for 1 h. Sometimes higher temperatures (> 100° C) are necessary. The derivatives were examined by TLC, the conditions being the same as those described for amines.

*Example for application.* Detection of ethanol in chloroform using benoxaprofen chloride. Chloroform (100 µl) was mixed with 500 µl of a solution of benoxaprofen chloride in dichloromethane (1 mg/ml). The mixture was heated to 60° C for 60 min. Then 10 µl of the reaction mixture were applied onto a TLC plate (silica gel 60) which was developed in solvent system II (without ammonia).

*Influences on the fluorescene intensity of benoxaprofen derivatives*

*Solvents, acids and bases.* Benoxaprofen-α-methylbenzylamide was applied onto TLC plates (100 ng/spot; at least two spots on each plate). The plates were developed in solvent system II (without ammonia) and dried completely. Each plate was half dipped into a tank containing one of the solvent mixtures A—K (see Table II for composition of solvents). Then the plates were dried in the air. The fluorescence intensity was measured at various times after dipping. The benoxaprofen-α-methylbenzylamide spots on the untreated part of the plates were used as references.

*Light and air.* The fluorescence of benoxaprofen-α-methylbenzylamide on

silica plates (100 ng/spot) was measured immediately after development and drying of the plate. Then one plate was exposed to light and air and a second plate was kept as a reference in vacuum and darkness (ambient temperature). The fluorescence intensity was measured twice a day over a period of four days.

## RESULTS AND DISCUSSION

### *Reaction products*

Numerous primary and secondary amines and aliphatic alcohols can easily be converted to well-detectable compounds by activated benoxaprofen.

TABLE I

$R_F$ VALUES AND RETENTION TIMES OF SEVERAL DERIVATIVES USING DIFFERENT STATIONARY AND MOBILE PHASES

Chromatographic conditions are described in the text. For composition of solvent systems I—V and VII, see section *TLC conditions*. For composition of HPLC solvent systems, see section *HPLC conditions*.

| Substance | $R_F$ values on TLC plates | | | | | Retention times in HPLC (min) | |
|---|---|---|---|---|---|---|---|
| | Silica gel 60 | | | RP-18 VII | | System a (silica gel) | |
| | I | II | III | | | | |
| α-Methylbenzylamine | 0.16 | 0.45 | 0.81 | 0.31 | | 6.7 | |
| | 0.28 | 0.56 | | | | 10.8 | |
| Tranylcypromine | 0.16 | 0.39 | 0.76 | 0.32 | | 9.0 | |
| | 0.21 | 0.45 | | | | 10.7 | |
| Amphetamine | 0.14 | 0.53 | 0.81 | 0.29 | | 8.0 | |
| | 0.21 | 0.59 | | | | 9.5 | |
| Methamphetamine | 0.27 | 0.65 | 0.79 | 0.23 | | 10.5 | |
| | 0.33 | | | | | 11.5 | |
| β-Phenylethylamine | 0.16 | 0.49 | 0.79 | 0.33 | | 11.8 | |
| Tolylethylamine | 0.18 | 0.49 | 0.79 | 0.30 | | 10.9 | |
| Phenylbutylamine | 0.18 | 0.48 | 0.80 | 0.26 | | 11.3 | |
| Benzylamine | 0.19 | 0.44 | 0.77 | 0.37 | | 10.3 | |
| Maprotiline | 0.25 | 0.69 | 0.82 | 0.13 | | 8.6 | |
| Procaine | 0.08 | 0.14 | 0.30 | 0.04 | | 8.5 | |
| Benoxaprofen | 0.09 | 0.05 | 0.68 | 0.43 | | 4.3 | |
| Benoxaprofen chloride | 0.64 | 0.84 | 0.85 | — | | 4.3 | |
| | Silica gel 60 | | | RP-8 V | RP-18 VII | System a (silica gel) | System b (RP-8) |
| | I | II | IV | | | | |
| Methanol | 0.69 | 0.44 | | | 0.32 | 11.4 | |
| Ethanol | 0.73 | 0.48 | | | 0.29 | 9.9 | |
| Propanol | 0.79 | 0.51 | | | 0.25 | 8.5 | |
| Butanol | 0.81 | 0.53 | | | 0.22 | 7.3 | |
| Isoamyl alcohol | 0.83 | 0.54 | | | 0.20 | 6.7 | |
| Choline | | | 0.36 | 0.21 | | | 7.5 |
| Scopolamine N-butylbromide | | | | 0.09 | | | 8.3 |
| Benoxaprofen | 0.09 | 0.05 | 0.86 | 0.45 | 0.48 | 4.3 | |
| Benoxaprofen chloride | 0.64 | 0.84 | 0.86 | 0.20 | | 4.3 | |

Benoxaprofen chloride and benoxaprofen imidazolide show good reactivity. The derivatives of amines can be prepared at room temperature in various dried solvents (e.g. dichloromethane, chloroform, acetonitrile, toluene).

Experiments were performed to optimize the reaction conditions. In the case of α-methylbenzylamine, the influence of bases on the reaction was investigated. Without bases the yield of reaction product with α-methylbenzylamine was about 91% after 1 h. If sodium or potassium carbonate or triethylamine (freshly distilled) was added, the yield was nearly 100%. After 12 h identical yields of reaction product (~100%) were obtained in all cases. If triethylamine was used as proton acceptor in the reaction mixture even trace amounts of contaminating ethylamine and diethylamine were detected and interfered in the determination of the substrates. Addition of carbonates increased the standard deviation and sometimes had an unfavourable influence on the TLC behaviour of the reaction mixtures. Therefore, in the case of α-methylbenzylamine and tranylcypromine, it is preferable to perform the derivatization without the addition of bases.

Reaction times can be shortened by heating the reaction mixture to 50—60°C. For the reaction with some drugs which contain OH groups, higher temperatures (e.g. 110°C) are sometimes necessary. The optimal reaction conditions (reaction time and temperature, addition of bases, solvent) for the derivatization with activated benoxaprofen have to be investigated for each substrate.

Examples of the formation of derivatives with benoxaprofen chloride and the chromatographic properties of the derivatives are given in Table I. Thin-layer chromatograms of the reaction mixtures of benoxaprofen chloride with five different alcohols ($C_1$—$C_5$) are shown in Fig. 2A and B.

For the development of analytical methods it is advantageous if highly purified reference substances are available in a sufficient amount. In general, derivatives of amines and alcohols with benoxaprofen can easily be synthesized

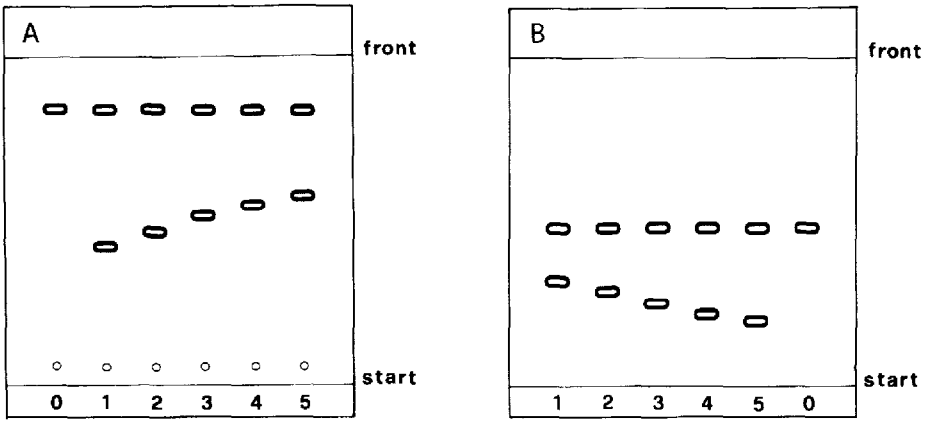


Fig. 2. (A) Chromatogram of benoxaprofen chloride reaction mixtures of five different alcohols from $C_1$ to $C_5$, directly applied to a silica gel plate (mobile phase: cyclohexane—dichloromethane—tetrahydrofuran (5:4:1, v/v), ammonia atmosphere). 0 = without alcohol (blank sample), 1 = methanol, 2 = ethanol, 3 = propanol, 4 = butanol, 5 = isoamyl alcohol. (B) Chromatogram of reaction mixtures of the five alcohols ($C_1$—$C_5$) on an RP-18 TLC plate (mobile phase: methanol—water (9:1, v/v)).

in adequate quantities. The benoxaprofen amides and esters of all the primary and secondary amines and alcohols investigated are strongly fluorescent. Amino acids and similar substances can also be detected in very low concentrations [4]. Even problematical substances such as, for example, quaternary compounds with reactive functional groups (e.g. choline, scopolamine N-butylbromide) can easily be changed into fluorescing, well-detectable compounds [5] which can be assayed by TLC and HPLC on silica gel or reversed-phase stationary phases.

*Fluorescence properties*

The fluorescence spectra of benoxaprofen after TLC or HPLC are shown in Fig. 3A and B. On TLC plates the excitation maximum is localized at 310 nm, the emission maximum at 365 nm. The spectrum registered during HPLC

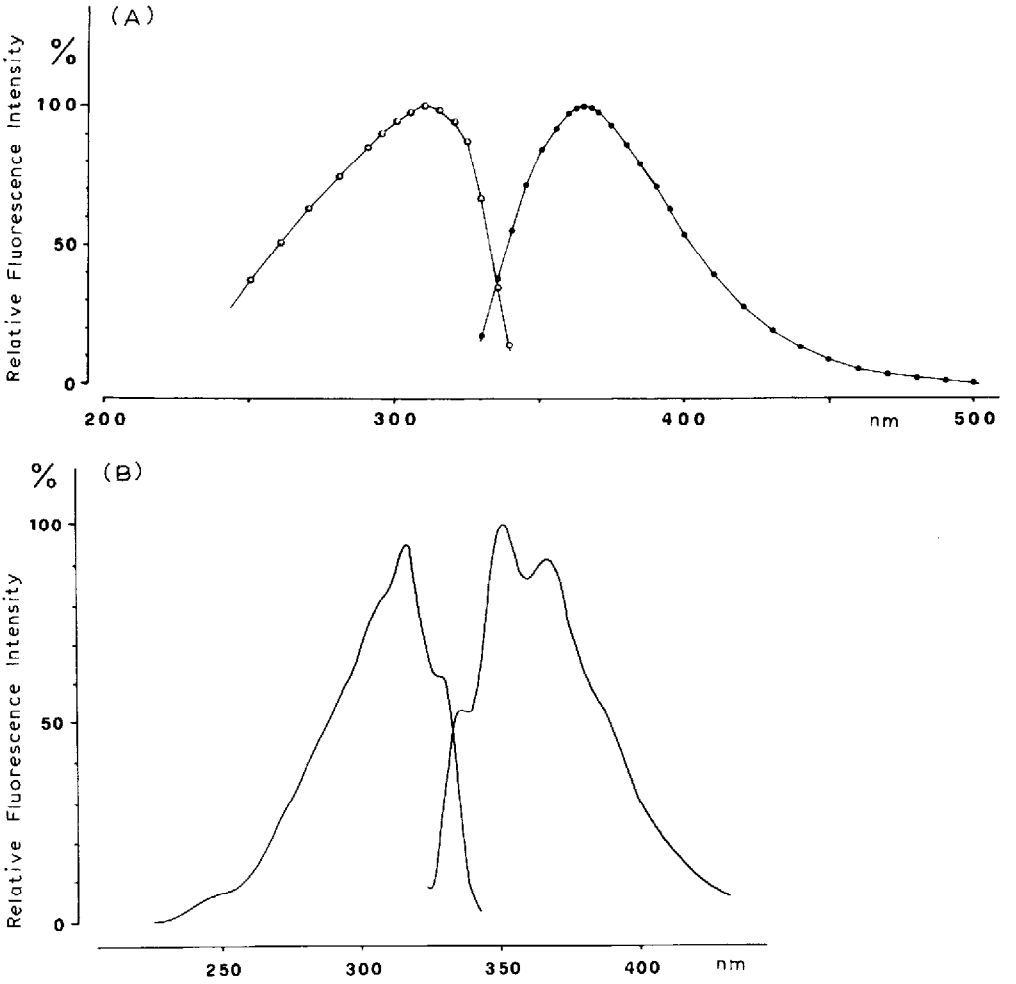


Fig. 3. (A) Fluorescence excitation and emission spectra of benoxaprofen on silica gel TLC plates. Excitation maximum = 310 nm, emission maximum = 365 nm. (B) Fluorescence excitation and emission spectra of benoxaprofen measured during HPLC. Excitation maximum = 312 nm, emission maxima = 350 and 365 nm.

shows an excitation maximum at 312 nm; there are two maxima in the emission spectrum, at 350 nm and at 365 nm. The spectra of benoxaprofen chloride are identical with the benoxaprofen spectra.

The quantum fluorescence yield for the benoxaprofen molecule was not determined. However, it may be supposed that it is in the same range as for related molecules, e.g. 2-(4-fluorophenyl)-5-phenyloxazole (quantum yield 0.95) [6].

All derivatives investigated exhibit fluorescence properties that are similar to or even identical with those of benoxaprofen or benoxaprofen chloride. In solution, i.e. during HPLC, the excitation and emission spectra of all these derivatives are nearly congruent with those of benoxaprofen. On TLC plates several but only insignificant hypsochrome or bathochrome shifts were found, which were obviously not due to different pH values of the solvent systems. Because of the high fluorescence intensity of benoxaprofen the detection limit on TLC plates is very low, namely 50—100 pg benoxaprofen per spot, i.e. 1/6 up to 1/3 pmol of benoxaprofen or benoxaprofen derivative.

*Detection of alcohols in organic solvents*

With benoxaprofen chloride it is possible to detect even small amounts of alcohols in organic solvents. Fig. 4 shows the TLC scans of benoxaprofen ethyl ester after the addition of benoxaprofen chloride to chloroform of different grades of purification.

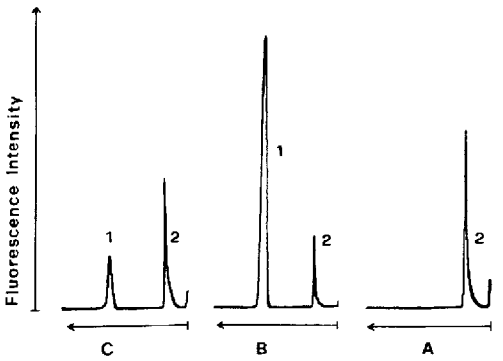


Fig. 4. TLC detection of ethanol in chloroform as benoxaprofen ethyl ester (= 1; benoxaprofen = 2): (A) blank sample; (B) chloroform p.a. (stabilized with ethanol); (C) chloroform for chromatography, stabilized with amylene.

*Derivatization after extraction from biological materials*

After derivatization with activated benoxaprofen it is possible to quantify, also in biological materials, very low concentrations of substances that in the underivatized state or after derivatization with other agents can be determined not at all or only in a high concentration range. Fig. 5 shows the TLC separation of maprotiline and one of its metabolites after extraction from plasma and derivatization. The calibration curve for maprotiline after derivatization with benoxaprofen chloride shows a linear relationship within the range 25—1000 ng/ml. Fig. 6 illustrates the HPLC separation of β-phenylethylamine extracted from plasma and derivatized to the corresponding amide.

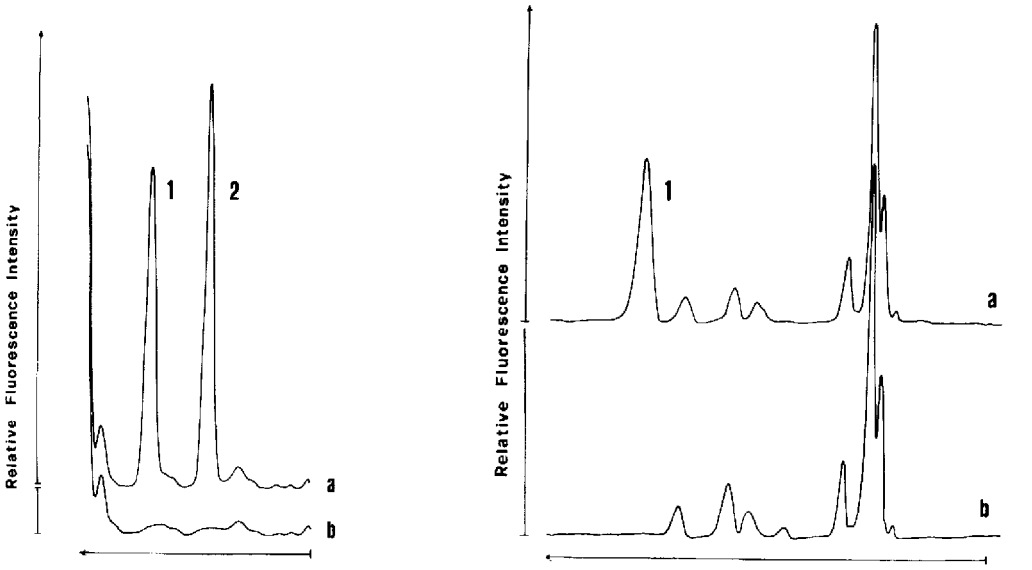


Fig. 5. Thin-layer chromatogram of maprotiline (1) and desmethylmaprotiline (2) after extraction from plasma (150 ng/ml) and derivatization with benoxaprofen chloride (= a; blank plasma = b). TLC separation was performed on silica gel plates using toluene—dichloromethane—tetrahydrofuran (5:2:1, v/v/v; ammonia atmosphere) as mobile phase.

Fig. 6. HPLC separation (system a) of β-phenylethylamine (1) after extraction from plasma (100 ng/ml) and derivatization with benoxaprofen chloride (= a; blank plasma = b).

*Influence on the fluorescence intensity of benoxaprofen derivatives (e.g. benoxaprofen-α-methylbenzylamide)*

*Solvents, acids and bases.* The detection limit can even be lowered by spraying the plates with viscous organic solvents [7]. Table II and Fig. 7 show the influence of dipping the dried plates into various solvents (neutral, acidic and basic). Mixtures containing paraffin as a component enhanced the fluorescence intensity markedly. The investigations also demonstrate that the plates must be dried completely before measuring, as volatile organic solvents also increase the fluorescence intensity.

TABLE II

INFLUENCE OF SOLVENTS, ACIDS AND BASES ON THE FLUORESCENCE OF BENOXAPROFEN-α-METHYLBENZYLAMIDE

The peak heights are related to the peak height on untreated plates, which is 100% or 1. The fluorescence intensity was measured 2 h after dipping.

| Solvent system | Relative peak height |
|---|---|
| Untreated plate | 1.00 |
| A. Paraffin—chloroform—isopropanol (1:1:4, v/v) | 2.49 |
| B. Paraffin—chloroform—isopropanol—concentrated ammonia (5:5:20:2, v/v) | 1.58 |
| C. Isopropanol | 1.00 |
| D. Isopropanol—concentrated ammonia (12:1, v/v) | 0.75 |
| E. Solution of sodium hydroxide (10%) in methanol—water (1:1, v/v) | 0.58 |
| F. Triethanolamine—chloroform—isopropanol (1:1:4, v/v) | 0.01 |
| G. Solution of citric acid (2%) in ethylene glycol—water—methanol (2:5:100, v/v) | 0.90 |
| H. Ethylene glycol—water—methanol (2:5:100, v/v) | 0.89 |
| I. Solution of citric acid (2%) in water—methanol (1:20, v/v) | 0.82 |
| K. Paraffin—chloroform—isopropanol—glacial acetic acid (5:5:20:2, v/v) | 2.89 |

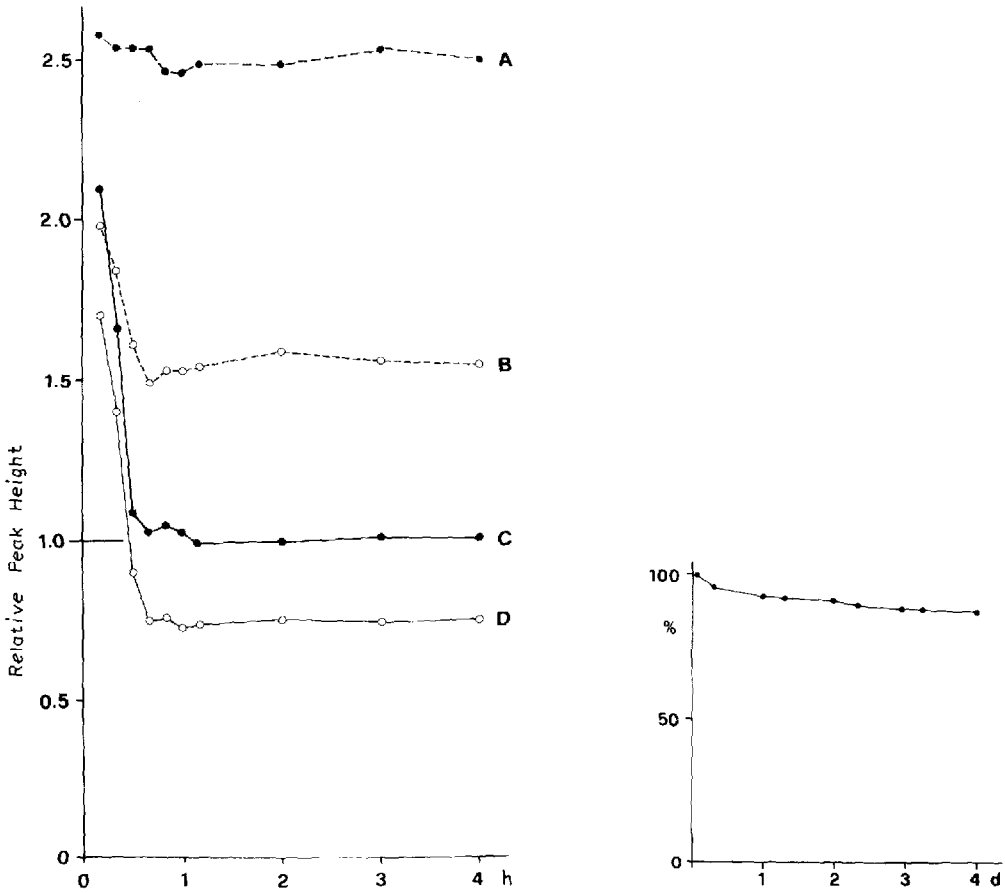


Fig. 7. Relative peak heights of benoxaprofen-α-methylbenzylamide on TLC plates within 4 h after dipping in solvent systems A—D, respectively. (The peak height on the untreated plates is 1.)

Fig. 8. Relative fluorescence intensity of benoxaprofen-α-methylbenzylamide on TLC plates exposed to light and air within 96 h. The values are means of four separate determinations. The standard deviations range from 0.9 to 2.5%.

*Light and air.* Fig. 8 shows the decrease of the fluorescence intensity within four days, if the plates are exposed to daylight and air. After 96 h the initial fluorescence intensity was diminished only by 13.5%.

*Chemical stability*

A special advantage of the fluorescence marker benoxaprofen chloride and benoxaprofen imidazolide is the chemical stability of the fluorophor. By-products have not been observed. Our experiments demonstrate that even at high temperatures (e.g. 110° C) the reaction takes place without any decomposition. The addition of potassium carbonate or acids to the reaction mixture does not influence the stability of the reagent or the products. In solution the derivatives are so stable that they are quantifiable even if stored for several days. Chromatography (TLC, HPLC) can be carried out in neutral as well as in acidic or basic eluents without decomposition.

*Conclusions*

In summary, it can be stated that benoxaprofen can easily be activated and that activated benoxaprofen is a reactive and an intensely fluorescent marker. The derivatives formed are stable. The spectral properties and chemical stability of the esters and amides on thin-layer plates as well as after HPLC separation allow their qualitative and quantitative analysis in the lower picomole range. This fluorescent label seems to be applicable for the development of sensitive assay methods for toxicological and pharmacological investigations. Furthermore, it was observed that after reaction of the activated racemic benoxaprofen with optically active compounds (e.g. α-methylbenzylamine, tranylcypromine), two peaks or spots with different retention times and $R_F$ values are obtained in several chromatographic systems. These peaks or spots could be identified as the respective diastereoisomers. In a previous paper the application of (+)- and (−)-benoxaprofen as reagents for the TLC and HPLC determination of chiral amines has already been described [8].

ACKNOWLEDGEMENTS

This work has been supported by the Dr. Robert Pfleger-Stiftung (Bamberg, F.R.G.) and the Deutsche Forschungsgemeinschaft.

The authors wish to thank Eli Lilly (Bad Homburg, F.R.G.) for the supply of adequate amounts of benoxaprofen and Dr. Liddiard for reading the manuscript.

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