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Note

Analysis of Benoxaprofen and other α-methylarylacetic acids using high-performance liquid chromatography

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Benoxaprofen, 2-(4-chlorophenyl)-α-methyl-5-benzoxazoleacetic acid (1), is a novel anti-inflammatory compound[1]. Enantiomers of several anti-inflammatory α-methylarylacetic acids are known to exhibit different biological activities and show preferential metabolism[2–7]. Consequently 1 was resolved as its quinine salt[8] so that the pharmacology and metabolism of the enantiomers could be studied. A chromatographic method was developed to support the resolution of 1 and to measure the purity of the resolved enantiomer[9].

Optimum chromatographic separation of enantiomers is usually obtained after conversion of the unresolved sample into a mixture of diastereoisomers[10–15]. An existing gas chromatographic method for the analysis of 1 using conventional phases requires high temperatures with resultant shortening of column life and gives poor resolution of the diastereoisomer peaks. Consequently an alternative high-performance liquid chromatography (HPLC) assay was developed.

The method can be applied to the analysis of further α-methylarylacetic acids and to the preparative separation of 1.

EXPERIMENTAL

Chemicals and reagents

The reagents A.R. thionyl chloride (Koch-Light Labs., Colnbrook, Great Britain), (+)- and (−)-α-methylbenzylamine (Aldrich, Milwaukee, Wisc., U.S.A.) and (+)-α-methoxy-α-trifluoromethylphenylacetic acid (Gold Label, Aldrich) were used as supplied. The HPLC solvents, methanol and methylene chloride, were purchased from Rathburn Chemicals (Walkerburn, Great Britain), whilst isooctane was obtained from BDH (Poole, Great Britain). The Hypersil APS was supplied by Shandon (Runcorn, Great Britain). (+)- and (−)-Benoxaprofen were resolved at the Lilly Research Centre. Ketoprofen (May & Baker, Dagenham, Great Britain) was used as supplied and Fenoprofen (Lilly Industries) after liberation of the free acid from the calcium salt.

Derivatization procedure

The α-methylarylacetic acid (1 mg) was reacted with thionyl chloride (100 μl) for 45 min at 80° in a reacti-vial. The resultant solution was blown to dryness under nitrogen and triethylamine (300 μl) and (—)-α-methylbenzylamine (20 μl) added. The solution was shaken and kept at 40° for 1 h before being blown to dryness. The residue was made up to 1 ml in the HPLC eluent and 20 μl injected onto the column.

Preparation of standard enantiomer mixtures

Solutions of both Benoxaprofen enantiomers (1 mg/ml) in methylene chloride were prepared. Aliquots from each were mixed to give a number of standard solutions which were evaporated to dryness to give enantiomeric mixtures (1 mg) of known composition.

Instrumentation

Chromatography was performed using a constant-flow Milton Roy Constametric IIG pump and a Cecil 212 variable-wavelength UV detector set at 308 nm. Samples were injected using a Rheodyne variable volume valve injector of Micromeritics auto-sampler. Data were either calculated following manual measurements of peak heights on a chart paper recorder, or by the use of a laboratory data system developed in the laboratories of Eli Lilly & Co. (Indianapolis, Ind., U.S.A.).

Chromatography

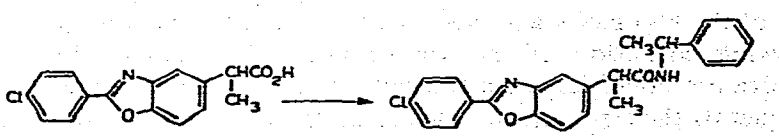
Separations were performed on a 12.5 cm × 5 mm I.D. 5-μm Hypersil APS stainless-steel column, packed vertically from an isopropanol slurry. A 1 ml/min flow-rate was maintained for the isooctane–methylene chloride–methanol (55:44.8:0.2) eluent. The Ketoprofen diastereoisomers were eluted under identical HPLC conditions although the solvent composition was modified to 75:24:1 for elution of the Fenoprofen diastereoisomers.

Determination of optical purity of α-methylbenzylamines

(+)-α-Methoxy-α-trifluoromethylphenylacetic acid (200 mg) was refluxed on a steam-bath with thionyl chloride (2 g) for 2 h and the solution evaporated to dryness. The residue was dissolved in methylene chloride (10 ml) and two 5-ml portions reacted with (+)- and (—)-α-methylbenzylamine (75 mg) at 25° for several hours. After removal of solvent the resultant amides were analyzed by HPLC using the Hypersil APS column and an isooctane–methylene chloride–methanol (75:25:0.5) eluent.

RESULTS AND DISCUSSION

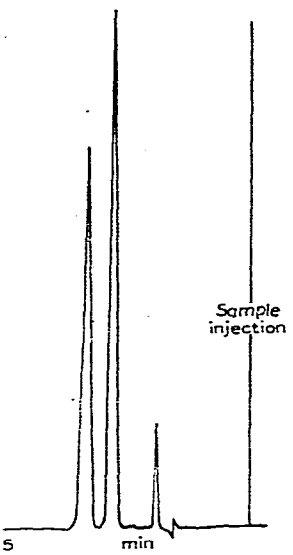
The Benoxaprofen enantiomers (1) were analyzed as their α-methylbenzylamides (2) following initial conversion to the corresponding acid chlorides. As in gas chromatography, optimum separation of diastereoisomers appears to be achieved



(1) (2)

when the functional group of both enantiomers and resolving agents are adjacent to the chiral centres[16]. As fortuitously α-methylarylacetic acids satisfy this criterion, it was hoped that the choice of (—)-α-methylbenzylamine as resolving agent would result in the formation of diastereoisomers showing good separation characteristics.

Although initial separation (resolution, $R_s = 3.85$) of the two diastereoisomers was achieved on a 12.5 cm × 5 mm I.D. column packed with 5-μm Hypersil, a small impurity coeluting with one of the diastereoisomer peaks interfered with the analysis. Consequently, a second separation (Fig. 1) was developed using 5-μm Hypersil APS, a chemically bonded aminopropyl silica packing material. Under these conditions no interference was observed but the peak resolution, R_s, was reduced to 1.59. The optical purity of (—)-α-methylbenzylamine was checked by reaction with the acid chloride of the optically pure reagent (+)-α-methoxy-α-trifluoromethylphenylacetic acid. Only the (—) (+) amide diastereoisomer was observed by HPLC confirming the amine was of high optical purity. One diastereoisomer was again observed following derivatization of the same acid chloride with (+)-α-methylbenzylamine. Interestingly, a mixture of these latter two diastereoisomers exhibited an R_s value of only 1.12 on HPLC using similar conditions to those used for the Benoxaprofen diastereoisomers. Hence the steric effect exhibited by the benzoxazole nucleus may be making a significant contribution to the separation of the diastereoisomers 2.



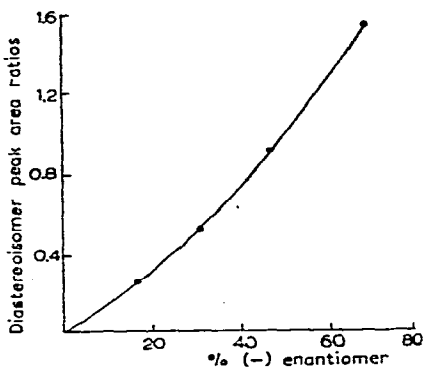


Fig. 1. Chromatogram showing separation of Benoxaprofen diastereoisomers on 12.5 cm × 5 mm I.D. 5-μm Hypersil APS column. Mobile phase, isooctane–methylene chloride–methanol (55:44.8:0.2). Flow-rate 1 ml/min.

Fig. 2. Graph correlating experimental diastereoisomer peak area ratios with sample composition.

The identity of the two peaks assigned to 2 were checked by solid-probe mass spectrometry following collection of the appropriate HPLC eluate. Even under electron-impact ionization conditions, benzoxazoles show minimal fragmentation of the nucleus. Consequently the resultant two mass spectra, which are very similar, show only three groups of ions corresponding to the molecular ion (*m/e* 404/6) and ions

corresponding to the loss of the amide group of *m/e* 256/8 $[M-CONCH\cdot CH_3\cdot C_6H_5]^+$ and *m/e* 257/9 $[M-OC=N\cdot CH\cdot CH_3\cdot C_6H_5[^+$.

Samples of (+)-Benoxaprofen ($[\alpha]^{22°} = +37.9 \pm 0.6°$ (0.4% w/v in ethanol)) and (−)-Benoxaprofen ($[\alpha]^{22°} = -37.5 \pm 0.6°$ (0.4% w/v in ethanol)) were purified to an optical purity of 98.6% and 98.2% respectively. The accuracy and precision of the method was checked from the analysis of blended mixtures of the pure enantiomers, the ratio of the areas of the diastereoisomeric peaks being used to give the experimental optical purities.

Impurities observed following the derivatization did not interfere with the analysis and a satisfactory correlation was observed between experimental and theoretical data (Fig. 2). The HPLC coefficient of variation was 0.3% for computer data acquisition and 1.2% for manual peak area ratio measurement (nine replicates).

As the Benoxaprofen enantiomers can be recovered without racemization by acid hydrolysis of the diastereoisomers, the approach can be used to resolve preparative amounts of enantiomer. However, the poor solubility of the diastereoisomers in the eluent may limit the scale of separation.

The method was successfully applied to Ketoprofen, and the free acid from Fenoprofen. It should be generally applicable to α-methylarylacetic acids. However, as expected the diastereoisomers from both these less bulky molecules were more difficult to separate. The Ketoprofen diastereoisomer pair (M^+, *m/e* 357) show an R_s value of 1.66 when analyzed under identical HPLC conditions to those used for the Benoxaprofen diastereoisomer. Using a modified solvent composition the Fenoprofen diastereoisomers (M^+, *m/e* 345) were eluted with a lower R_s value of 1.37.

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