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Study of the stereochemistry of ethambutol using chiral liquid chromatography and synthesis

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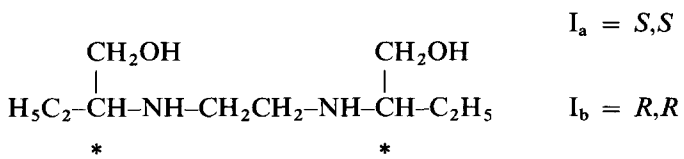
ABSTRACT

Ethambutol [N,N-ethylenebis(2-aminobutan-1-ol)dihydrochloride] is shown to have *S,S* absolute stereochemistry by unambiguous synthesis of each of its possible stereomers and chiral chromatography, using Pirkle π interaction chiral stationary phases. Separation of the *R,R*; *S,S* and *R,S* stereomers together with separation of the entantiomers of 2-aminobutan-1-ol, as their perbenzoyl derivatives, is described. The method is used to study therapeutic tablets. Spectroscopic data is used to characterise these derivatives.

INTRODUCTION

Ethambutol is the approved name [1] for the (+)isomer of N,N-ethylenebis(2-aminobutan-1-ol) dihydrochloride (hereafter referred to as the diamine I). It is an important drug [2] used in the treatment of tuberculosis and was introduced by Lederle and marketed under their trade name of Myambutol.

Examination of the structure of the diamine I shows that three stereomers (I_a–I_c) are possible depending on the absolute stereochemistry of the two chiral (*) centres. I_a and I_b are "enantiomers" whilst the third isomer is an optically inactive *"meso"* form.



Diamine I

I_a = *S,S*

I_b = *R,R*

I_c = *R,S*

Ethambutol must be prescribed with care [3,4] because of its known visual-disturbance side effects, which may progress to irreversible optic nerve damage. These side effects are associated, to an equal extent, with each of the stereomers (I_{a-c}) [5].

However, the beneficial antibacterial activity [6,7] is markedly dependent upon the stereomer used. The (−)isomer is virtually inactive whilst the (+)isomer is 12 times

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more active than the *meso* isomer. For this reason one "pure" enantiomer (+) is used therapeutically and its specific rotation $[\alpha]_D$ has been specified in official monographs [8–10]. [British Pharmacopoeia (BP), United States Pharmacopoeia (USP), European Pharmacopoeia (EP).]

Early monographs (*e.g.*, BP 1973) only specified the optical activity of ethambutol but this was changed in the 1980 BP to define the absolute stereochemistry of (+)ethambutol as *R,R*. Similar changes took place in the USP and the EP. In contrast, the stereochemistry of (+)ethambutol is shown as *S,S* in Klyne's Atlas [11].

However,the USP XXI (1985) changed the stereochemical designation to *S*-(*R**,*R**) and the BP (1988, amendment No. 3) changed the stereochemistry to *S,S*.

The EP (1987) defined the stereochemistry of ethambutol as *R,R* and also introduced an assay based on the optical rotation of a copper complex, measured at the mercury wavelength 436 nm. This was quite different from the polarimetry studies of the sodium D wavelength 589 nm, used in BP and USP monographs. Clearly there has been serious confusion concerning ethambutol.

We now report studies of the diamine I, using chiral chromatography and synthesis, which permit independent analysis of the three stereomers and confirm the *S,S* absolute stereochemistry of the therapeutic stereomer, (+)ethambutol.

EXPERIMENTAL

Material

Ethambutol hydrochloride was purchased from Sigma (Poole, U.K.). Ethambutol tablets (400 mg) were obtained from the Pharmacy dispensary, Barnsley District General Hospital, Barnsley, U.K. *R*-(−)-2-Aminobutan-1-ol (97%) was purchased from Fluka. *S*-(+)-2-aminobutan-1-ol (98%), racemic 2-aminobutan-1-ol (97%), benzoyl chloride (99%) and 1,2-dibromoethane (99%) were purchased from Aldrich (Gillingham, U.K.). All solvents used were HPLC grade, other materials were of laboratory grade and used as purchased.

Equipment

The high-performance liquid chromatographic (HPLC) system used contained an LKB (Bromma, Sweden) 2150 pump and a Rheodyne (Berkeley, CA, U.S.A.) injection valve (Model 7125) with a 20-μl loop. Two chiral columns were used, a (25 cm × 4.9 mm I.D., 5 μm particle size) Pirkle covalent bonded N-3,5-dinitrobenzoyl derivative of D-phenylglycine (Regis, Morton Grove, IL, U.S.A.) and a (25 cm × 4 mm I.D.) Nucleosil Chiral-2 (Macherey-Nagel, Düren, F.R.G.). A variable-wavelength UV detector (DuPont, Stevenage, U.K.) set at 230 nm and a Hewlett-Packard 3388A integrator–printer/plotter were employed. The mobile phase flow-rate was set at 1 ml/min in all studies but two different phase compositions were used, as indicated in the text. Mobile phases were prepared by premixing the required proportions of solvents, by volume, and were used without further degassing or filtration. An in-line filter (2 μm) was fitted between the solvent reservoir and the pump.

IR spectra were run on a Perkin-Elmer 297 instrument. Electron impact mass spectrometry (EI-MS) was carried out on an AEI MS902 instrument, using a direct-insertion probe and electron-impact source (250°C; 70 eV ionising energy), equipped with a Mass Spectrometry Services data system.

Synthesis of the three diamine stereomers of ethambutol

R-(−)-2-Aminobutan-1-ol was reacted with 1,2-dibromoethane, converted to the hydrochloride salt of *R,R*-ethambutol and crystallised from ethanol as outlined [7]. An analogous reaction was carried out using *S*-(+)-2-aminobutan-1-ol to yield the *S,S*-ethambutol isomer.

When the same reaction was carried out using racemic 2-aminobutan-1-ol, a mixture of products was obtained from which the *meso* (*R,S*) isomer of ethambutol selectively precipitated as its free base, as outlined [7]. The contents of the mother liquors were converted to a crystalline hydrochloride salt which was examined by chiral HPLC.

Preparation of perbenzoyl derivatives of individual ethambutol stereomers, micro-derivatisation (general method)

The required diamine (1–2 mg) as its free base or hydrochloride salt was weighed into a quickfit centrifuge tube and sodium hydroxide (2 *M*, 3 ml) was added followed by benzoyl chloride (0.1 ml). The tube was stoppered and swirled for at least 8 min. More benzoyl chloride (0.025 ml) was added and the stoppered tube was shaken for a further 7 min. The mixture was then extracted with dichloromethane (3 × 3 ml) and the pooled extracts were dried over sodium sulphate, filtered and reduced to dryness using a rotary evaporator. The residue was dissolved in hexane–propan-2-ol (10 ml, 75:25, v/v) and examined by chiral HPLC.

This method was used to perbenzoylate ethambutol (Sigma) and ethambutol extracted from a tablet with dichloromethane. Synthetic samples of *S,S*-diamine, *R,R*-diamine, *R,S*-diamine (*meso*) and racemic diamine (*R,R*/*S,S* equal mixture) were also individually derivatised and examined by chiral HPLC.

Subsequent scale up produced sufficient perbenzoylated product for each isomer to permit examination by MS and IR spectroscopy. None of the derivatives could be obtained in crystalline form.

Preparation of the dibenzoyl derivative of R-(−)-2-aminobutan-1-ol (AMB), micro reaction

R-(−)-2-Aminobutan-1-ol (0.04 ml) was pipetted into a volumetric flask (100 ml), dissolved and adjusted to volume with dichloromethane. A sample (1 ml) was transferred to a quickfit centrifuge tube and reduced to dryness with a stream of dry nitrogen. Sodium hydroxide (2.5 *M*, 1 ml) was added to the tube, followed by benzoyl chloride (0.025 ml) and the tube was stoppered and swirled for at least 10 min. The mixture was then extracted with dichloromethane (3 × 3 ml) and the pooled organic layers were dried over sodium sulphate, filtered and reduced to dryness under reduced pressure. The residue was dissolved in hexane–propan-2-ol (12 ml, 9:1, v/v) and examined by chiral HPLC.

Preparation of the dibenzoyl derivatives of S-(+) and racemic AMB

Each sample of AMB was, in turn, derivatised using the micro reaction described for *R*-(−)-AMB above. The derivatives produced were examined by chiral HPLC.

These reactions were scaled up to produce sufficient material from each isomer to enable mass spectrometry and infra red characterisation. Each product was obtained in crystalline form.

RESULTS AND DISCUSSION

Many methods of chiral HPLC analysis have been reported and recently reviewed [12] but few permit the separation of purely aliphatic enantiomers. Usually at least one aromatic ring is required to give the necessary "3-point interaction". In contrast, aliphatic amino acids have been separated by chiral gas chromatography (GC) [13,14].

Our initial approach was to examine ethambutol, its stereomers and its synthetic precursor AMB as both their free bases and as their trifluoroacetyl derivatives [15,16] using a XE-60–*S*-valine-*S*-α-phenylethylamide chiral capillary GC column (Chrompack). The trifluoroacetylation procedure and the free base extraction methods used were as reported [16] in the literature. The chiral column was tested using racemic N-trifluoroacetyl amino acid-*n*-butyl esters [17].

Achiral GC, using both packed and capillary columns, was used to monitor the extraction and derivatisation stages, prior to examination of the free bases and their trifluoroacetyl derivatives on the chiral GC column.

Chiral GC results were, in our hands, disappointing because we could not detect any separation of racemic AMB, either as its free base or its trifluoroacetyl derivative. The chiral column was shown to be in working order as it gave baseline separation for each derivatised racemic amino acid (Ala, Val, Leu). With ethambutol no peak could be eluted, either as free base or trifluoroacetyl derivative, because of the low thermal stability of the chiral column (maximum operating temperature 190°C).

Our next approach was to study chiral HPLC. We initially chose Pirkle-type columns since they are some of the less expensive chiral columns, were in use already in our laboratory and are relatively robust. A prerequisite of such studies was derivatisation. Perbenzoyl derivatives were made since they contained both an aromatic ring chromophore to enable UV detection and amide/ester groups capable of dipole stacking and so producing strong, enantioselective column interactions. These derivatives were only examined on two different π-interation chiral stationary phases (CSPs) because of cost limitations. Preliminary results were encouraging so reference compounds were synthesised and examined.

Synthetic procedures

Individual isomers (*S,S* and *R,R*) of the diamine were prepared by reacting *S*-(+)-AMB or *R*-(−)-AMB respectively with 1,2-dibromoethane, as outlined by Wilkinson *et al.* [7].

The absolute stereochemistry of each AMB isomer was specified in the supplier's catalogue and we assumed they correspond to the chemical transformation reactions from *S*-(−)-methionine outlined in Klynes and Buckingham's work [11].

An analogous reaction was carried out using racemic AMB. Part of the yield crystallised first as a relatively insoluble free-base, corresponding to the *R,S-"meso"*-diamine isomer as reported by Wilkinson and co-workers [6,7]. The remainder of the yield was subsequently obtained as a crystalline hydrochloride. This was essentially a racemic mixture (equal *R,R* and *S,S* isomers) contaminated with a variable amount of *"meso"* isomer.

Derivatisation procedure

The same general reaction (Schotten Baumann) was used to perbenzoylate each of the above synthetic diamines and mixtures of diamine stereomers, as well as starting AMB stereomers and commercial ethambutol.

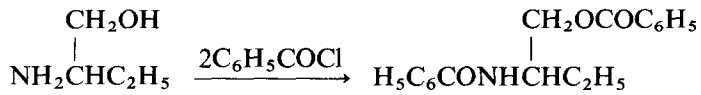
The nitrogen base or its hydrochloride was dissolved in aqueous alkali and then swirled with excess benzoyl chloride at room temperature. If the benzoyl derivatives were in sufficient quantity and crystalline they were collected by filtration. If not, they were extracted into dichloromethane. The organic solvent was dried, removed under reduced pressure and the residue was dissolved in appropriate mobile phase prior to HPLC.

Spectroscopic characterisation of perbenzoyl derivatives

The perbenzoyl derivative of racemic AMB was obtained as a crystalline product (m.p. 102–104°C). Its IR spectrum (KBr disc) showed two carbonyl absorbances at wavenumbers 1630 cm^{-1} (amide group) and 1710 cm^{-1} (ester) together with a singlet N–H absorbance at 3300 cm^{-1}.

Its low-resolution EI-MS showed a weak (M^+) molecular peak at m/z 297.

These data are consistent with the dibenzoylation reaction shown below.



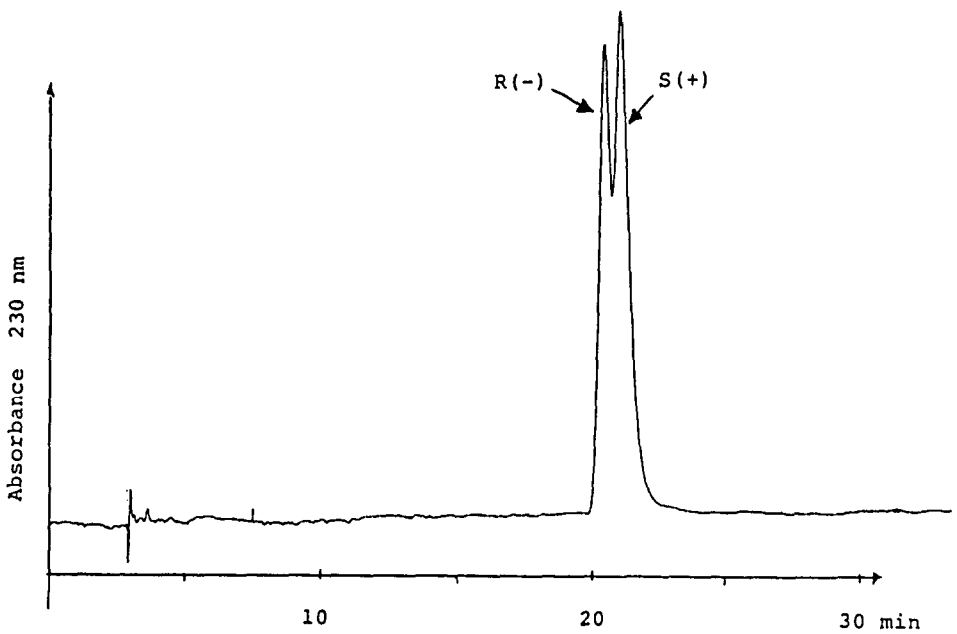


Fig. 1. Separation of enantiomers of 2-aminobutan-1-ol, as perbenzoyl derivatives on a Pirkle D-phenylglycine column. Mobile phase, hexane–propan-2-ol (9:1, v/v). Flow-rate, 1 ml/min. Equivalent to 5 μg of free base "on column".

A similar crystalline product (m.p. 112–113°C) was obtained when *R*-(−)-AMB was similarly treated. Its IR and MS spectra were almost identical to those of the racemic derivative. However, both these dibenzoyl derivatives could be clearly distinguished when studied by chiral chromatography (see Figs. 1 and 2).

When ethambutol dihydrochloride (Sigma) was perbenzoylated in a similar manner a non-crystalline product was obtained. Its IR spectrum (chloroform solution) showed two carbonyl absorbances at wavenumbers 1720 cm^{-1} (ester) and 1630 cm^{-1} (amide). No N–H band at 3300 cm^{-1} was observed.

Its low-resolution EI-MS was not definitive because a very weak and hard-to-measure molecular peak was observed at *m/z* 619. However, significant fragment peaks were observed at *m/z* 591 (M − C_2H_5), 515 (M − C_6H_5CO) and 485 (M − $CH_2OCOC_6H_5$). Work using NMR and milder ionisation methods (chemical ionization, fast atom bombardment) is in hand to rigorously establish the structure of this derivative.

The above spectroscopic data are consistent with the tetrabenzoylation reaction shown below.

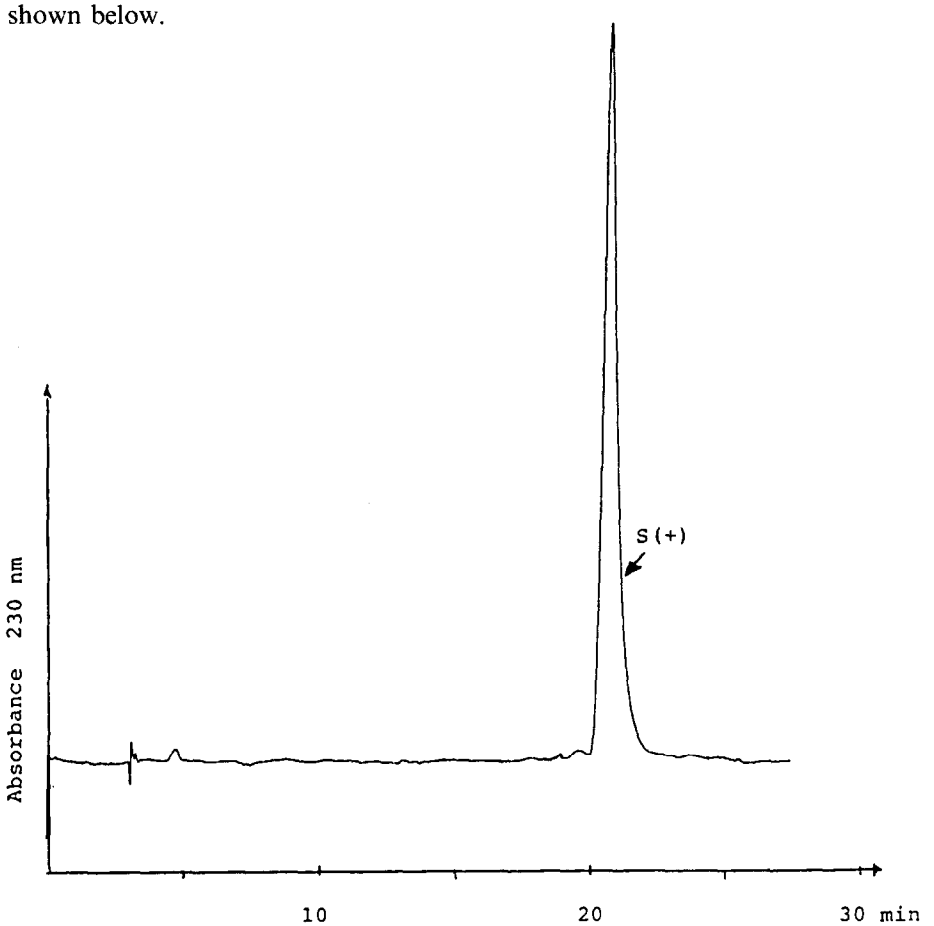
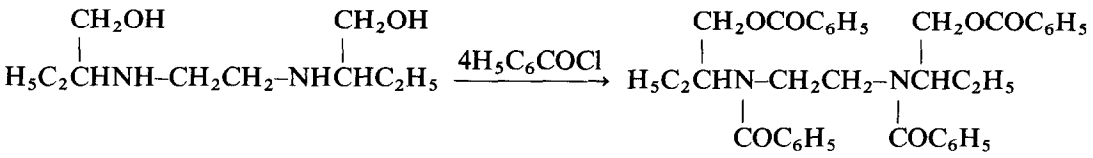


Fig. 2. Commercial sample of *S*-(+)-2-aminobutan-1-ol, as its perbenzoyl derivative on a Pirkle D-phenylglycine column. Mobile phase, hexane–propan-2-ol (9:1, v/v). Flow-rate, 1 ml/min. Equivalent to 5 μg of free base "on column".



Similar perbenzoylation reactions were carried out starting with our synthetic samples of *S,S*-; *R,R*-; *R,S*-(*meso*)- and racemic (*S,S*/*R,R*)-diamines. All these derivatives were obtained in non-crystalline form and their MS were very similar.

However, all stereomers could be distinguished when examined by chiral HPLC (see Figs. 3 and 4).

Micro derivatisation

To facilitate chromatographic studies and as a preliminary to developing a usable assay, a simplified microderivatisation procedure, involving no crystallisation stages, was developed. This was applied to each of the commercial or synthetic materials discussed above as well as being used to study ethambutol in tablets. In this way samples (*ca.* 1 mg) of AMB or diamine isomers could be rapidly examined.

Chiral chromatography

Two commercial chiral columns were used in isocratic mode (hexane–propan-2-ol, 75:25, v/v). The first was a Pirkle column containing covalently bound, 3,5-dinitrobenzoyl-D-phenylglycine (Regis). The second was a Machery-Nagel column, Nucleosil Chiral 2 but the manufacturers regard this as proprietary and supply no

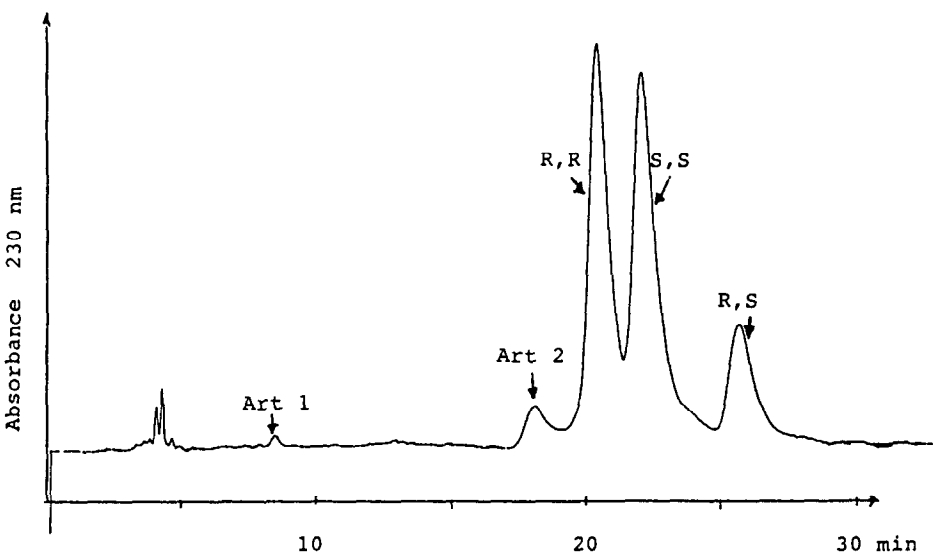


Fig. 3. Separation of a mixture of N,N-ethylenebis(2-aminobutan-1-ol) stereomers and reaction artefacts, as perbenzoyl derivatives on a Pirkle D-phenylglycine column. Mobile phase, hexane–propan-2-ol (75:25, v/v). Flow-rate, 1 ml/min. Equivalent to 1–3 μg of free base "on column".

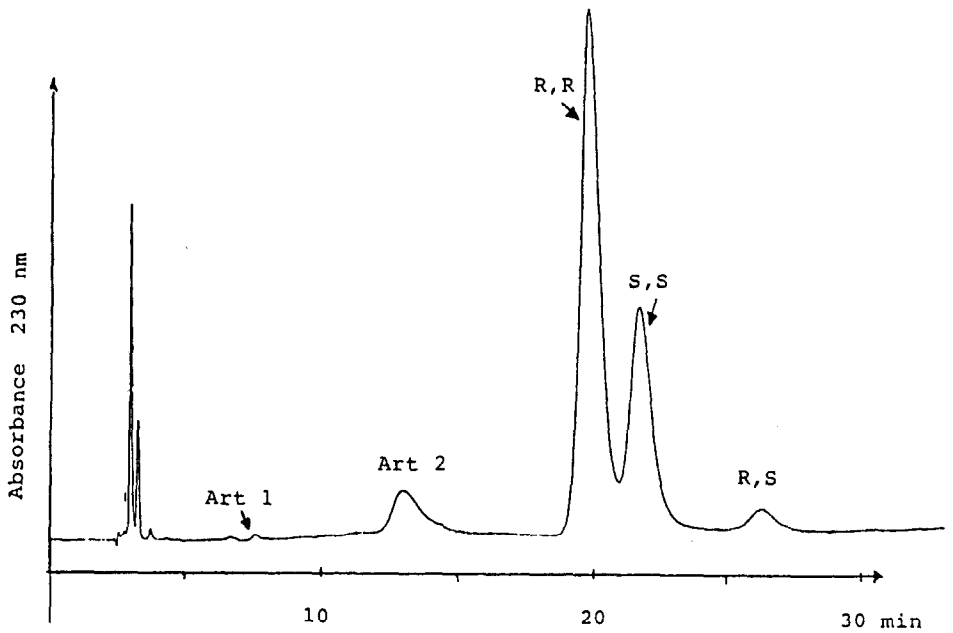


Fig. 4. Separation of a mixture of N-ethylenebis(2-aminobutan-1-ol) stereomers and reaction artefacts, as perbenzoyl derivatives on a Macherey-Nagel Nucleosil Chiral 2 column. Mobile phase, hexane–propan-2-ol (75:25, v/v). Flow-rate, 1 ml/min. Equivalent to 1–3 μg of free base "on column". For peaks Art 1 and Art 2, see text.

details of its chiral ligands or preparation. In previous studies of herbicides we had found this column to behave similar to the Pirkle column.

Both these columns behaved similarly towards the derivatised diamine stereomers (but not amino butanols, see later) and gave separation of all three isomers (see Figs. 3 and 4). By using individual stereomers it was possible to assign the absolute configuration to each elution peak and show that the *R,R*-isomer eluted first, the *S,S*-isomer next and the *R,S-meso*-isomer last. This elution order was the same on both columns.

Samples of ethambutol (Sigma) and ethambutol extracted from a medicinal tablet were microderivatised separately and then shown to correspond to the *S,S*-isomer. In both cases minor impurity peaks could be detected corresponding to the *R,S-meso*-isomer, but no peak corresponding to the *R,R*-isomer was detected. It was noticed during some micro derivatisation runs that two groups of minor peaks sometimes eluted before the diamine derivatives. One (Art 1) appears to arise from AMB contaminating the diamine, particularly some of our synthetic samples. This peak was identified by spiking samples with dibenzoyl AMB. The other (Art 2) peak probably arises from incomplete perbenzoylation since its appearance depends upon the time allowed for derivatisation. The approximate retention time for these artefacts is indicated on the chromatograms (Figs. 3 and 4).

Under the isocratic conditions described for diamine HPLC analysis no separation of the dibenzoyl derivative (Art 1) of racemic AMB was observed.

However, when the mobile phase was changed to hexane–propan-2-ol (90:10, v/v) partial resolution of this dibenzoyl derivative was found using the Pirkle column (see Figs. 1 and 2). The elution order was checked using authentic samples of *R*-(−)-AMB and *S*-(+)-AMB. The derivatised *R*-(−)-isomer eluted first. Surprisingly no resolution on the Nucleosil Chiral 2 column could be obtained. Unless Macherey-Nagel supply further details of its mode of action this column can only be used in an empirical manner.

Both columns gave reproducible separations and were used over several months without obvious deterioration in performance.

CONCLUSION

Unambiguous characterisation of the three stereomers of N,N-ethylenebis(2-aminobutan-1-ol) can be accomplished by perbenzoylation and chiral chromatography, using a covalent Pirkle column. More work is required to validate the method before it can be used for stereochemical and chemical assay of ethambutol.

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