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Note

Chromatographic separation of β-substituted 3-(3,4-dihydroxyphenyl)alanine derivatives

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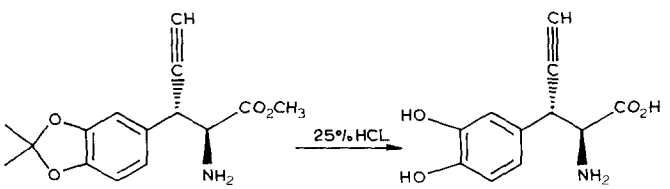
Hydroxyphenylalanine compounds such as *ortho*-, *meta*- and *para*-tyrosine, dihydroxyphenylalanine and iodotyrosine derivatives have been analysed either by ion-exchange [1] or high-performance liquid chromatography (HPLC) [2–4]. As these molecules show significant absorption at wavelengths between 240-280 nm, they can be detected at low concentrations by spectrometric detection without the need for derivatisation [5–7].

In the present study we report the reversed-phase HPLC analysis of a number of closely related β-substituted dihydroxyphenylalanine derivatives. This chromatographic method was investigated as a possible final purification step in the isolation of (2*S*,3*S*)-2-amino-3-(3,4-dihydroxy phenylalanine)-4-pentynoic acid (**2**) which was prepared from the corresponding methyl ester (**1**). The dihydroxy phenylalanine derivative (**2**) was required as part of a program aimed at the design of mechanism based inhibitors of dopamine β-hydroxylase [8].



(1) (2)

EXPERIMENTAL

Chemicals

Compound **1** was synthesised by the Medicinal Chemistry Section of SmithKline Beecham (Welwyn, U.K.). All reagents used in the synthesis of **2** from **1** were purchased from Aldrich (U.K.) and were used without further purification.

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Water used in HPLC analysis was double-distilled deionised quality. Methanol, acetic acid, formic acid and phosphoric acid were AnalaR grade purchased from BDH (U.K.). Trifluoroacetic acid was obtained from Aldrich.

Acid hydrolysis of 1

A solution of **1** (461 mg, 1.67 mmol) and L-ascorbic acid (5 mg, 0.11 mmol) in 5 ml of 6.9 *M* aqueous hydrochloric acid was heated under argon at 80–90°C for 3 h, then at 115°C for 1.5 h. After cooling the solution was loaded on to an ion-exchange column (600 × 30 mm) packed with a strongly acidic cation-exchange resin [Bio-Rad 50W-X2 (H)] and **1** was eluted with 1 *M* aqueous pyridine. All fractions containing materials active to ninhydrin after spot testing on a thin-layer chromatography plate (LiChrosorb RP-8; BDH) were combined and solvent was removed under reduced pressure. The ion-exchange process was repeated two more times. After removing the eluting solvent, the residue was dissolved in water and the solvent removed under vacuum. This process was repeated another time and finally the residue was dissolved in water and freeze dried to leave a cream-coloured solid (265 mg) which was analysed by HPLC.

HPLC

Chromatographic analysis was performed on a Perkin-Elmer Series 4 liquid chromatograph equipped with a Perkin-Elmer ISS-100 autoinjector. A Kratos Spectroflow 783 variable-wavelength absorbance detector was set at 280 nm.

Reversed-phase analysis was carried out on a μBondapak C_{18} column (30.0 × 3.9 mm I.D., 10 μm) supplied by Millipore (Sweden). Four mobile phases were used with this column. Each contained 2.5% methanol as one of the components. The remaining 97.5% consisted of one of the following four acids: acetic (0.0164 *M*, pH 3.01), formic (0.0217 *M*, pH 2.28), phosphoric (0.0102 *M*, pH 1.83) and trifluoroacetic (0.0088 *M*, pH 1.73). These molarities correspond to solutions of these acids in water in the ratio of 0.1 to 99.9 (v/v).

Mass spectral characterisation

MS data were obtained with a Finnigan TSQ46 mass spectrometer, mass range 125–650 a.m.u. The vapouriser temperature was set at 120°C. Ammonium acetate (0.1 *M*) was added before detection.

RESULTS AND DISCUSSION

The acid hydrolysis of the methyl ester **1** gave four major products, denoted by peaks A–D in Fig. 1, and a number of minor impurities. The chromatographic separation of the four major components was found to be best when either 0.1% trifluoroacetic acid or 0.1% phosphoric acid was used in the mobile phase. In contrast, 0.1% acetic acid or formic acid gave shorter retention times and a decrease in the resolution of peaks B and C. In all cases the addition of 2.5% methanol as the cosolvent in the mobile phase was necessary to give sharp peaks. 0.1% trifluoroacetic acid was chosen as the mobile phase in preference to phosphoric acid as this acid had no adverse effects on the mass spectral identification of components A–D.

The mass spectra obtained for compounds A–D are shown in Fig. 2. None of the

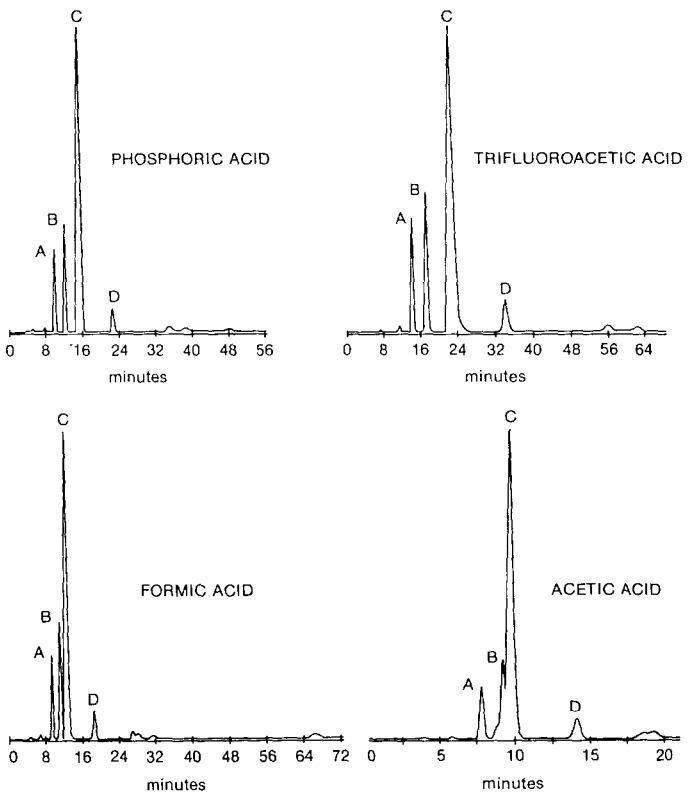

Fig. 1. HPLC analysis of the reaction mixture from the hydrolysis of **1** using 0.1% (a) phosphoric acid, (b) trifluoroacetic acid, (c) formic acid and (d) acetic acid in the mobile phase. For peaks A–D, see text.

molecules corresponds to starting material **1**. Peak D was identified as compound **5** which is a product from the partial hydrolysis of **1** (m/z 262 $(M+1)^+$, 234, 218). The dioxan ring in **5** is still intact. Peak C indeed corresponds to the desired amino acid **2** (m/z 222 $(M+1)^+$), whereas peaks A and B are due to isomeric compounds **3** and **4** (m/z 240 $(M+1)^+$, 223; 196) which arise from the addition of a water molecule across the acetylenic bond of either **1** or **2**.

The order of elution of compounds **2** to **5** is related to their hydrophobicity. Thus compound **5** which lacks the two hydroxyl groups on the aromatic moiety has the longest retention time. The ethynyl group in **2** makes this molecule more hydrophobic than **3** or **4** which have a keto or an aldehyde substituent in the β-position of the amino acid group. Peaks A and B were tentatively assigned to the structures **3** and **4** shown as **3** is expected [9] to be slightly more hydrophobic than **4**.

The pK_1 and pK_2 values of dihydroxyphenylalanine have been reported as 2.31 and 8.71, respectively [10]. The corresponding pK values of **2**–**5** are expected to be lower than those for dihydroxyphenylalanine due to the electron-withdrawing nature of the substituents in the β-position of the amino acid groups in these compounds. Under the acidic conditions used to separate these molecules the amino group is

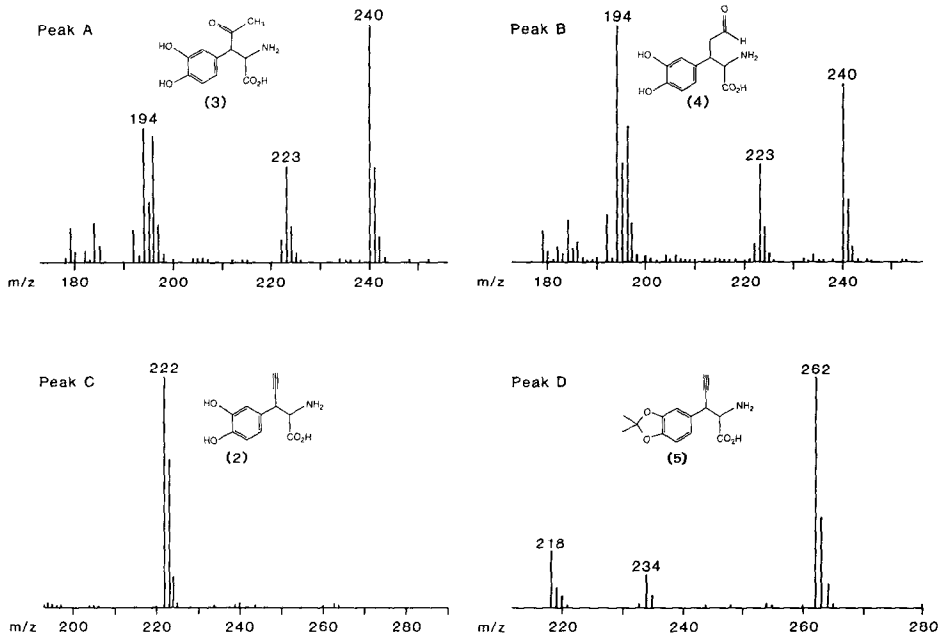

Fig. 2. Mass spectral characterisation of compounds corresponding to peaks A–D (see text). Stereochemistry has been omitted from the structures.

protonated in all cases. The pK_a value of the carboxyl group is around 2 so that its extent of ionisation to the carboxylate form will be low at pH values lower than 2. However, it is only in the case when either 0.1% trifluoroacetic acid (pK_a = 0.25) or 0.1% phosphoric acid (pK_a = 2.15) is used in the mobile phase that the pH of the eluent is lower than 2. As both acetic (pK_a = 4.76) and formic (pK_a = 3.75) are weaker acids than phosphoric or trifluoroacetic the pH of the mobile phase containing 0.1% of the former two acids is higher than the pK_a of the carboxylic group in compounds **2**–**5**, giving rise to longer retention times and poorer resolution.

In conclusion, 0.1% trifluoroacetic acid in the eluent has allowed facile separation of **2** from other components obtained in the hydrolysis of **1**. As the preparation of analytically pure **2** for biological testing proved to be difficult by other purification methods such as crystallisation or column chromatography, the HPLC method outlined was suitable for scaling up using a preparative column of the same stationary phase to prepare a chromatographically pure sample of **2** for biological testing.

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