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ORIGINAL RESEARCH

A simple chiral high-performance liquid chromatographic method to study the enantiomer-differentiating action of microorganisms. An assay with DL-tryptophan

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ABSTRACT: To investigate the 'enantiomer-differentiating' action of the microorganisms colonizing a phosphate-buffered DL-tryptophan solution, a novel chiral high-performance liquid chromatographic (HPLC) arrangement was developed and established. As the HPLC stationary phase, bovine serum albumin (BSA) bonded silica gel was used. In the function of the mobile phase, phosphate-buffered DL-tryptophan solution was applied. The composition of the eluate was monitored by an HPLC spectrophotometric detector. After injecting the assayed sample into the eluent stream, the content of each tryptophan enantiomer was evaluated either from the positive or negative responses of the HPLC detector. The validity and the performance of this novel approach were confirmed by applying another chiral HPLC device working with human serum albumin (HSA) bonded silica gel as the stationary phase and with 1-propanol containing phosphate buffer as the eluent. Copyright © 1999 John Wiley & Sons, Ltd.

INTRODUCTION

Recently we introduced a principally original high-performance liquid chromatographic (HPLC) arrangement for drug quality control (Šoltés *et al.*, 1996). The novelty in the method is that the drug, classified as the reference sample, is in the HPLC eluent. The injected sample contains the drug preparation assayed, whose solution was prepared by a procedure identical to that used with the reference drug run in the HPLC mobile phase. Any compositional difference between the reference and the drug sample analysed is easily recognizable from the HPLC detector response. Nil output signal at the retention time of the drug proves that the drug concentration is identical in the two samples. Any positive/negative peak detected means that the drug content in the sample assayed is different from that in the mobile phase. The validity of the method has already been demonstrated by analysing the chemical purity of ibuprofen batches (Šoltés *et al.*, 1996).

The aim of the present paper is to demonstrate the utility of the proposed HPLC arrangement in investigating the enantiomer-differentiating action of the microorganisms colonizing a phosphate-buffered DL-tryptophan solution.

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EXPERIMENTAL

Materials and chemicals

The DL-, D- and L-tryptophans used were products of Sigma Chemical Co. (St Louis, MO, USA). KH_2PO_4 and $Na_2HPO_4 \times 12H_2O$, both of p.a. purity grade, were supplied by Merck, Darmstadt, Germany. Water was of Milli-Q_{RG} quality (Water Purification System; Millipore Corporation, Bedford, MA, USA). The phosphate buffer (0.067 mol/L, pH 7.4) as well as the phosphate-buffered DL-tryptophan (stock) solution (2.0×10^{-4} mol/L) were cleansed immediately before use by filtration through a cellulose acetate membrane with 0.45-μm pores (Type DE 67; Schleicher and Schuell, Dasel, Germany). Prior to loading into the HPLC device, the samples analysed were passed through a syringe filter with 0.20-μm pores (3 mm i.d.; Sun International Trading, Ltd, Wilmington, NC, USA).

HPLC apparatuses

The HPLC device **1** comprised a mobile phase delivery pump (Model 422; Kontron Instruments S.A., Yvelines, France), a sample injection valve equipped with a 10/(100)-μL loop (Model 7125; Rheodyne, Cotati, CA, USA), and a variable-wavelength photometric detector (Model Spectra 100; Spectra-Physics Inc., Autolab Division, San Jose, CA, USA) set at 280 nm. A short stainless-steel column (4.6 mm × 5 cm) packed with LiChrospher

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Si 300 (Merck; mean particle size 10 μm) was inserted between the HPLC pump and the sample injection valve to saturate the mobile phase with SiO_2.

The separations were performed at ambient temperature by a tandem of a guard column (4.0 mm × 3 cm), packed with Resolvosil® (Macherey Nagel, Düren, Germany), and a Resolvosil® BSA-7PX column (4.0 mm × 15 cm; Macherey-Nagel). The mobile phase, run at a flow rate of 0.5 mL/min, was phosphate-buffered solution of DL-tryptophan (1.0×10^{-4} mol/L).

The eluent reservoir bottle as well as the teflon connecting tubes were protected against daylight by wrapping them in aluminum foil. During the measurements the reservoir bottle was immersed in a water–ice bath whose temperature did not exceed +5°C.

The second HPLC apparatus (**2**), constructed of the same modules as the above-described HPLC equipment (**1**), operated with a tandem of a chiral-HSA guard column (3.0 mm × 1 cm; ChromTech AB, Hägersten, Sweden) and an HPLC column (4.6 mm × 15 cm) packed with HSA-bond silica gel (mean particle size 10 μm; porosity 200 Å, Kromasil; Eka Nobel AB, Bohus, Sweden, content of the *in situ* bound protein = 95.8 mg HSA per 1 g of the silica gel). The mobile phase, run at a flow rate of 0.5 mL/min, was a bicomponent mixture of the phosphate buffer and 1-propanol, 97.0:3.0 (v/v). All chromatographic separations were performed at ambient temperature.

Samples

To calibrate both HPLC systems (**1, 2**), freshly prepared phosphate-buffered DL-, D- and L-tryptophan solutions, each of 2.0×10^{-4} mol/L, were used. Further calibrants were prepared from these three stocks and from the blank phosphate buffer closely before their loading into the HPLC device.

The samples investigated for the enantiomer-differentiating action of the microorganisms originated from the phosphate-buffered DL-tryptophan solution (1.0×10^{-4} mol/L) used as the eluent at working with the HPLC arrangement **1**. At different time intervals (cf. Fig. 2, conditions a–c), an aliquot of the solutions was ultrafiltrated and analysed on using both HPLC devices (**1, 2**).

RESULTS AND DISCUSSION

To saturate (and equilibrate) quickly the chiral stationary phase used on operating the HPLC device **1**, at the onset of the procedure a highly concentrated phosphate-buffered DL-tryptophan solution (1.0×10^{-3} mol/L) was loaded in 10 subsequent samples (10 × 100 μL) into the apparatus. In this way the chromatographic system attained the equilibrium state within less than half an hour.

Figure 1, record B, illustrates the (nil) detector response monitored after injecting an aliquot (10 μL) of the mobile phase into the equilibrated HPLC device **1**. Figure 1 (records A and C) shows the resolution of the HPLC arrangement **1** attained on injecting the blank phosphate buffer (record A) and the stock phosphate-buffered DL-tryptophan solution (record C). The retention times found were 2.5 and 4.5 min for D- and L-tryptophan, respectively. The calculated enantioselectivity factor (α) was 2.75. The high peak symmetry (cf. Fig. 1, records A and C) seen on operating this HPLC arrangement (**1**) can be considered a welcome bonus. The stability of the retention times as well as the linearity of the calibration curves found ($r^2 > 0.9999$) justify the application of this HPLC arrangement (**1**) for both qualitative and quantitative analysis of samples regarding their D- and/or L-tryptophan content(s).

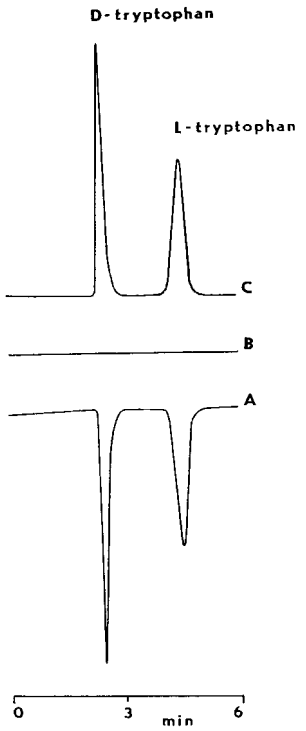

Figure 1. Chromatograms obtained by using the HPLC arrangement **1**. Samples loaded were blank phosphate buffer (A), aliquots of the eluent (B) or of the stock phosphate-buffered DL-tryptophan solution (2.0×10^{-4} mol/L) (C).

Figure 2 presents the time dependences of the [L-tryptophan]/[D-tryptophan] concentration ratios in the sample aliquots stored under various conditions: (a) daylight, ambient temperature; (b) daylight, cooling (≤ +5°C); (c) darkness, cooling (≤ +5°C). The results obtained indicate that by keeping the eluent reservoir bottle in a water–ice bath (at ≤ +5°C) and by eliminating any illumination, a constant ratio (50:50) of the two tryptophan enantiomers can be warranted in the mobile phase for at least one working day period [Fig. 2(c)]. This finding was confirmed on continuous monitoring of the composition of the mobile phase used with the HPLC device **1**, accomplished by the application of the second chiral-HPLC apparatus (**2**). The chromatogram shown in Fig. 3 is representative of any one of the records obtained at sampling the phosphate-buffered DL-tryptophan eluent during a working day.

In conclusion, it can be stated that at ambient

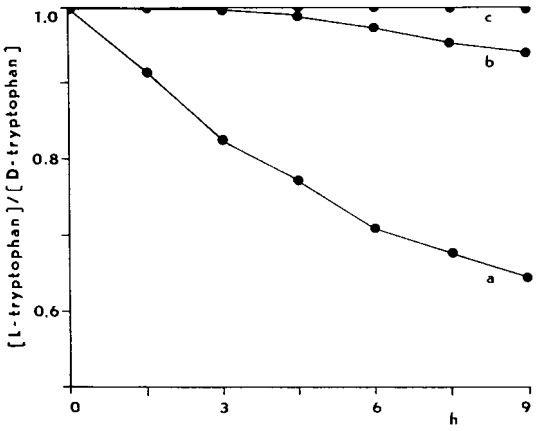

Figure 2. Time dependences of the [L-tryptophan]/[D-tryptophan] concentration ratios in the mobile phase reservoir. The mobile phase storage conditions were: (a) daylight, ambient temperature; (b) daylight, cooling ($\leq +5°C$); (c) darkness, cooling ($\leq +5°C$).

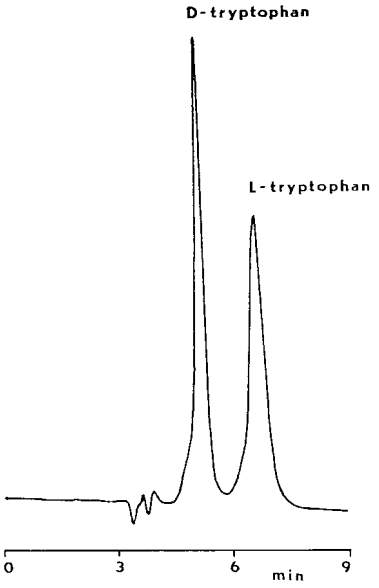

Figure 3. Chromatograms obtained by using the chiral-HPLC device **2**. Sample analysed was an aliquot (20 μL) of the mobile phase used on operating the HPLC device **1**.

temperature and under daylight a significant change occurs in the ratio of the two tryptophan enantiomers within a relatively short period of time [cf. Fig. 2(a)]. This so-called enantiomer-differentiating action of microorganisms (Allenmark *et al.*, 1986) was due to their rapid growth and preferential catabolism of the biogenic L-tryptophan amino acid. Preventive measures aiming at exclusion of this process appear to be mandatory on working with any (phosphate-buffered) DL-, D- or L-tryptophan containing solutions–either as samples (Sébille and Thuaud, 1980; Lagercrantz *et al.*, 1981; Erlandsson *et al.*, 1986), mobile phases in liquid chromatography (Yang and Hage, 1993; 1996; 1997), or running buffers in capillary electrophoresis (Hage and Tweed, 1997).

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