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Application of preparative liquid chromatography to the isolation of enantiomers of a benzodiazepinone derivative

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ABSTRACT

The isolation of milligram amounts of the enantiomers of a benzodiazepinone derivative was performed on an analytical cellulose tribenzoate-based column by multiple repetitive injections. An enantiomeric purity greater than 98% was required. First, an analytical method was developed to maximize the resolution by adjusting the mobile phase composition, flow-rate and most importantly the column temperature. Then the preparative separation was optimized by adjusting the sample size and detecting the sample where its UV absorbance was low. The locations of the cut points were determined by use of detector response levels. The method development, preparative separations and analytical assays of the fractions obtained were all performed on analytical columns.

INTRODUCTION

There are added complications in the preparative chromatography of chiral compounds compared with other small molecules because of the higher cost of the packing material, the large number of chiral packing materials, the low loadability of chiral columns [1], the strong temperature dependences [2,3] and often the limited choice of compatible mobile phases. Low loadability is typically due to limited ligand-binding capacities. Temperature increases can accelerate the kinetics of mass transfer but, depending on the combined effect of chiral selectivity and chromatographic efficiency, the resolution may increase or decrease [4].

There are three primary approaches to isolating milligram amounts of substances in elution chromatography: (1) employ an analytical column, typically 25 cm × 4.6 mm I.D. packed with 5- or 10-μm material, and perform hundreds of repetitive injections to isolate the compounds of interest [5]; (2) employ a large column, typically 25 cm × 2 cm I.D. packed with 10–20-μm particles, and make only a few injections to isolate the desired amount [6,7]; or (3) employ medium-pressure technology using columns packed with 40–60-μm materials and in one or two runs to isolate the desired amount of substance at the desired purity [8,9]. For each of these approaches there is a trade-off between the cost of the packing material, the cost of the preparative chromatograph, the time to perform the separation and in some instances the time required to make large amounts of stationary phase together with packing and testing columns. For approaches (2) and (3) a method is also required to determine the purity of the collected fractions. The first approach will consume the smallest amount of solute during method development and will minimize the loss of solute due to unforseen effects such as irreversible adsorption. In this work, the first approach was used in view of the availability of automated analytical equipment, good stability of the column packing material, the high cost of large columns and the amount of solute available.

Theoretical models have been used to predict overloaded elution profiles for several chiral systems with extremely high accuracy [10]. Several op-

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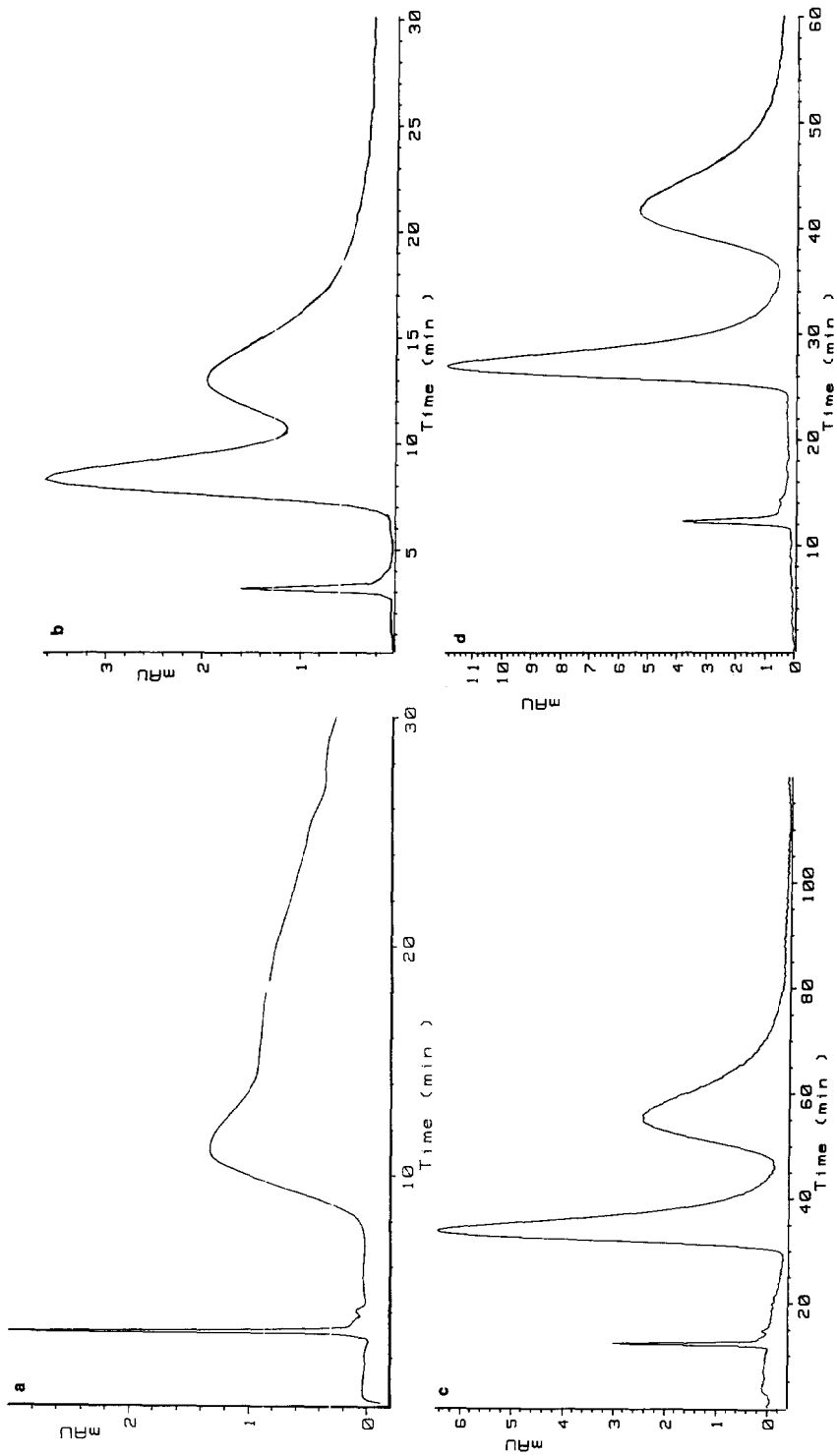

Fig. 1. Optimization of the analytical method, 1.0 μg of the solute in 10 μl of *n*-hexane–2-propanol (50:50, v/v) was injected on to the column in the Hewlett-Packard LC system. UV detection at 230 nm. (a) Flow-rate 1.0 ml/min, 22°C, *n*-hexane–2-propanol (50:50, v/v); (b) flow-rate 1.0 ml/min, 40°C, *n*-hexane–2-propanol (50:50, v/v); (c) flow-rate 0.25 ml/min, 40°C, *n*-hexane–2-propanol (50:50, v/v); (d) flow-rate 0.25 ml/min, 45°C, *n*-hexane–2-propanol (40:60, v/v).

timization theories have been presented with different assumptions [11–13]. The application of some of these concepts is considered in this paper.

This paper illustrates a practical example of the optimization of the experimental conditions for the isolation of a pair of enantiomers. First an analytical method was developed in order to improve the resolution by adjusting the eluent composition, the flow-rate and the temperature. Then the production rate was increased and optimized by increasing the sample size.

EXPERIMENTAL

Analytical system

Both analytical and preparative separations were performed on a Chiralcel OB 250 × 4.6 mm I.D. column obtained from Daicel Chemical Industries (Tokyo, Japan). This chiral column contains cellulose tribenzoate coated on 10-μm silica. Two 250 × 4.6 mm I.D. Chiracel OB columns were employed, one for the preparative purification and one for the analytical work. The chromatographic set-up for analytical method development consisted of a Hewlett-Packard (Waldbronn, Germany) Model 1090 M liquid chromatograph equipped with a ternary solvent delivery system and a diode-array detector, all controlled by a Hewlett-Packard ChemStation. Some analytical experiments were also performed on the preparative Kontron system (see below) which was replumbed for analytical purposes.

Preparative system

A Kontron (Zürich, Switzerland) MT2 data acquisition system was employed to control a Kontron Model 420 high-performance liquid chromatographic (HPLC) pump and a Model 425 low-pressure gradient former. The outlet of the pump was connected to a Valco (Houston, TX, USA) six-port pneumatic valve, then to the Chiracel OB column placed in a column thermostat from Hengeler Analytik-Instrumente (Riehen, Switzerland) maintained at 40°C. The column outlet was connected to a Kratos (Ramsey, NJ, USA) Spectroflow 783 UV detector and then to the second six-port valve on the Gilson (Villier Le Bel, France) Model 232/401 sample processor [14]. Repetitive large-volume injections for the preparative isolations were made by sending a sequence of ASCII codes [15] from the Kontron data system to a Hamilton (Bonaduz, Switzerland) Microlab M dispenser which aspirated and dispensed the sample into the loop of the Valco valve. The position of the Valco valve was controlled by contact closures.

The equipment used is specified in the figure captions as either Hewlett-Packard LC or Kontron LC. The collected fractions were evaporated to dryness in a Büchi (Flawil, Switzerland) Rotavapor RE. Resolutions (R_s), separation factors (α) and capacity factors (k') were calculated as described previously [16]. Reduced plate heights (h) were calculated using the peak width at the baseline. The column void volume was determined by injecting 20 μg of 1,3,5-tri-*tert.*-butylbenzene in 20 μl of *n*-hexane–2-propanol (95:5, v/v) using *n*-hexane–2-propanol (90:10, v/v) as the mobile phase at flow-rate of 1.0 ml/min at *ca.* 22°C.

Chemicals

1,3,5-Tri-*tert.*-butylbenzene was purchased from Aldrich-Chemie (Steinheim, Germany) and HPLC-grade *n*-hexane and 2-propanol from Fluka (Buchs, Switzerland) and used as received. The racemic benzodiazepinone derivative was obtained as a research sample from Ciba-Geigy (Basle, Switzerland).

RESULTS AND DISCUSSION

There are a large number of chiral stationary phases prepared by adsorption of cellulose derivatives on macroporous silica supports [17]. These phases shows good peak symmetry and efficiency; additionally, relative high loading capacities have been reported [18]. Therefore, the commercial columns selected for this work were those packed with cellulose tribenzoate coated on macroporous silica.

The selection of eluents was limited as only *n*-hexane modified with 2-propanol or ethanol was recommended. Moreover, relatively small changes, such as changing the eluent modifier from 2-propanol to ethanol or methanol, cause irreversible changes in the retention characteristic of the column. The mobile phase composition was optimized by testing eluents of increasing concentration of 2-propanol. The retention of the solute was observed to decrease with increasing amount of 2-propanol in the mobile phase.

The flow-rate and temperature were optimized

TABLE I

SUMMARY OF CHROMATOGRAPHIC DATA

1.0 mg of the solute in 10 ml of *n*-hexane–2-propanol (50:50, v/v) was injected on to the column in the Hewlett-Packard LC system. UV detection at 230 nm.

n-Hexane–2-propanol (v/v)	Flow-rate (ml/min)	Temperature (°C)	k'_1	a	R_s	h_1	h_2	Fig.
50:50	1.0	22	2.8	1.8	<0.4	>1000	>2000	1a
50:50	1.0	40	1.9	1.9	0.7	320	950	1b
50:50	0.5	40	1.8	1.9	1.2	140	340	
50:50	0.25	40	1.9	2.0	1.5	98	210	1c
40:60	0.25	40	1.5	2.0	1.5	87	180	
40:60	0.25	45	1.3	2.0	1.6	57	140	1d

consecutively. Fig. 1 illustrates selective chromatograms obtained during the optimization of these two parameters. Fig. 1a shows the separation of the racemic mixture under analytical conditions, 1.0 μg of sample in 10 μl of *n*-hexane–2-propanol (50:50, v/v) at room temperature (*ca.* 22°C) at a flow-rate of 1 ml/min. On increasing the temperature to 40°C, holding all other parameters constant, the selectivity was hardly affected and the apparent column efficiency increased (Fig. 1b). In order to increase further the column efficiency and thus the resolution, the flow-rate was reduced to 0.25 ml/min. Lastly, the conditions which resulted in the highest resolution (1.6) were *n*-hexane–2-propanol (40:60, v/v) at a flow-rate of 0.25 ml/min and 45°C (Fig. 1d). The chromatographic data describing these analytical results are summarized in Table I.

According to the manufacturer, the maximum operating temperature was 40°C; therefore, in order to minimize degradation of the column under conditions of large sample sizes, all the preparative operations were run at 40°C. The analytical separation factor under these conditions was 1.9–2.0. In order to increase the production rate, the sample size was increased by increasing the injection volume at a constant sample concentration of 0.5 mg/ml. Fig. 2 illustrates the chromatograms obtained at sample sizes of 0.5, 0.75 and 1 mg; however, in order to prevent detector saturation, the wavelengths monitored were 230, 295 and 300 nm, respectively. In view of the high purity requirements and in an effort to complete the isolation without recycling or reprocessing the collected fractions, the sample size chosen was 0.75 mg per injection.

The fraction cuts were made on the basis of the detector response levels, rather than time. Therefore, if degradation of the separation or shifts in retention time occurred, the purity of the fractions could be maintained. Fig. 3 illustrates the cut points. The solid vertical lines indicate the location of hard cuts, made on a time basis at 17 and 79 min. The horizontal line indicates the detector response cut-off level, which was made at 900 mV. Between 17 and 79 min when the detector response increased above 900 mV or dropped below 900 mV, a contact closure signal was sent to the sample processor,

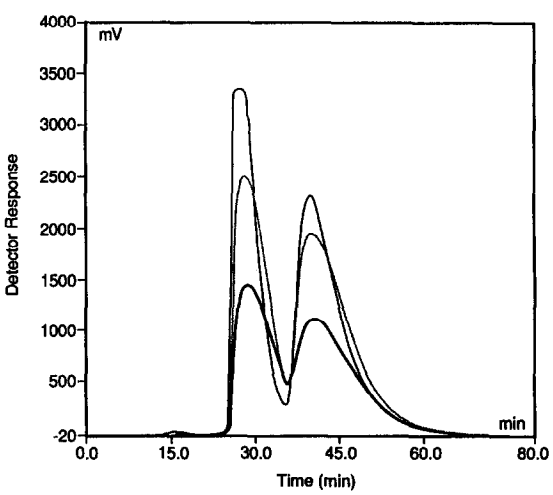

Fig. 2. Optimization of the sample size. Mobile phase, *n*-hexane–2-propanol (40:60, v/v); flow-rate, 0.25 ml/min; solute concentration, 0.5 mg/ml; Kontron LC system. Injection volume: thin solid line, 1.0 ml; dotted line, 1.5 ml; thick solid line, 2.0 ml. Detection wavelength: thin solid line, 230 nm; dotted line, 295 nm; thick solid line, 300 nm.

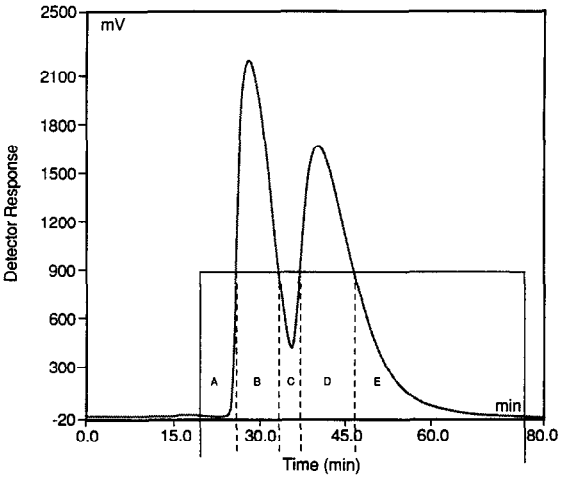

Fig. 3. Preparative chromatogram with cut points. Flow-rate, 0.25 ml/min; mobile phase, *n*-hexane–2-propanol (40:60, v/v); temperature, 40°C; UV detection at 295 nm; injection volume 1.5 ml; solute concentration, 0.5 mg/ml; Kontron LC system.

causing the fraction to be collected in the next free vessel. The dashed vertical lines indicate the expected location of the cuts taking into account the dead volume the solvent traverses between the detector outlet and the free vessel.

Reproducibility of the preparative chromatograms over a 2-day period including a change of sample solution is shown in Fig. 4. Excellent reproducibility was observed. Despite covering the sample, slight increases in the amount injected were observed owing to evaporation of the sample solvent over time.

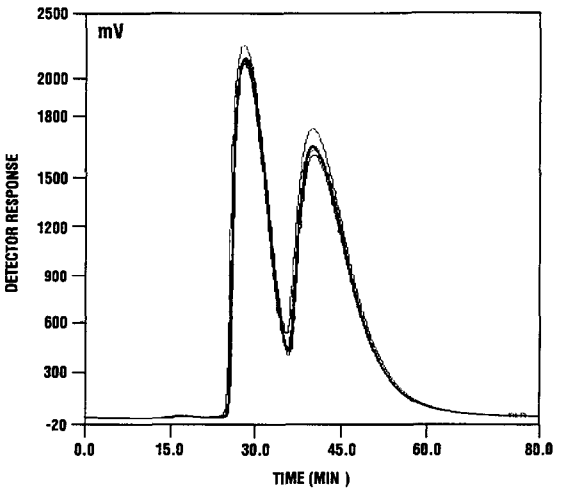

Fig. 4. Reproducibility of preparative chromatogram over 2 days. Experimental conditions as Fig. 3.

Analysis of the fractions gave the following results. Fraction A contained a negligible amount of the early-eluting enantiomer. Fraction B was collected at >99% enantiomeric purity. Fraction C was a mixture of both enantiomers. Fraction D contained about 7% and fraction E 5% of the early-eluting enantiomer. Fig. 5 shows the chromatogram of fraction B (dashed line) overlayed with the chromatogram of fraction D (solid line).

After 7 days of continuous operation, 17 mg of the first-eluting enantiomer were obtained at the purity required. However, in order to increase the purity of the later eluting enantiomers, fractions D and E were mixed, concentrated and reprocessed. This second preparative step was performed under the same experimental conditions as the first step. Fig. 6 shows a preparative chromatogram where 0.75 mg of total sample per run was injected. Similarly, cuts were made on the basis of detector response levels, 800 mV. The inset in Fig. 6 shows the analysis after fractions B2 and C2 had been mixed. The enantiomeric purity of the later eluting enantiomer was 98% and 19 mg were obtained. The second chromatographic step needed about 100 h of continuous operation.

When the analysis of the fractions were performed on a second chromatograph simultaneously with the preparative separations, the whole project, including method development, preparative chromatography and assay of fractions, was accomplished in 3 weeks.

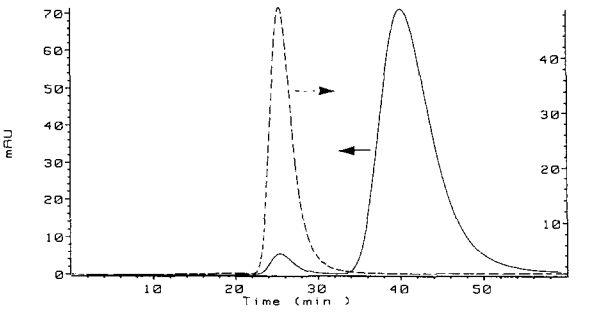

Fig. 5. Test of enantiomeric purity by analysis of the fractions. Mobile phase, *n*-hexane–2-propanol (40:60, v/v); flow-rate, 0.25 ml/min; temperature, 45°C; UV-detection at 230 nm; Hewlett-Packard LC system. Dashed line, fraction B; solid line, fraction D.

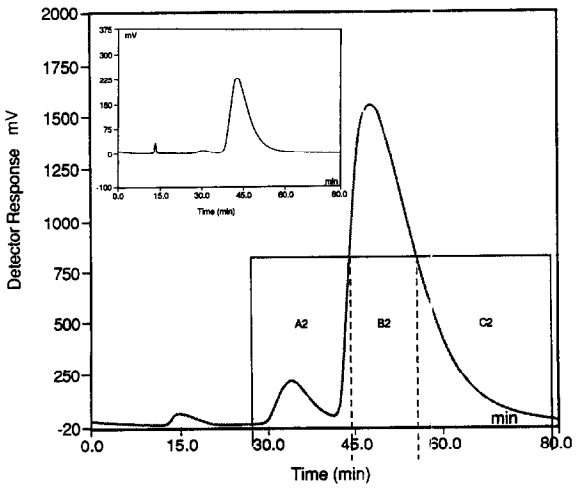

Fig. 6. Preparative chromatogram, second step. Experimental conditions as Fig. 3. Inset: test of enantiomeric purity for the second pass, fraction B2 + C2. Experimental conditions as Fig. 5, except temperature 40°C, but on the Kontron analytical system.

To achieve the isolation with a single preparative injection, assuming the same linear velocity, column length, particle size and chromatographic efficiency, we would have needed a 3.8 cm diameter column.

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