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Note

Comparison of two β-cyclodextrin bonded stationary phases for high-performance liquid chromatography

Elution order and optical purity of enantiomers of cyclohexylphenylglycolic acid

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In pharmacological and biological studies using optical antipodes it is very important that the optical purity of the test compound is known. Especially when the difference in biological activities between the enantiomers is large (ratio > 50), small optical impurities may lead to erroneous conclusions. In these cases it is necessary that the optical purity can be determined accurately in the range between 95 and 100%. However few techniques are available that can meet this demand.

The determination of the specific optical rotation is often not sufficient. The true value is not always known and, in general, the method it is not sensitive enough to deal with impurities of less than 2–3% (ref. 1). Nuclear magnetic resonance (NMR) techniques have been described for the determination of optical purity. Using chiral shift reagents, optical impurities could be determined if more than 5% impurity was present in samples of levamisole/dexamisole and methadone[2,3]. The smallest detectable optical impurity in a tertiary amine was found to be 1% by ^{1}H NMR of the diastereomeric salts of the amine with Mosher's reagent[4]. In various cases chromatography has proven to be a better alternative in the determination of optical impurities. Some papers have been published on enantiomeric resolution and subsequent determination of the optical purity by thin-layer chromatography. In some instances optical impurities down to about 0.25% could be detected[5]. However, gas chromatography (GC) and high-performance liquid chromatography (HPLC) are more suitable for the detection of trace amounts of optical impurities[6–8].

Recently, we described[9] an HPLC system for the resolution of enantiomeric aromatic carboxylic acids. The system consisted of a chiral stationary phase, containing β-cyclodextrin covalently bonded to silica gel (synthesized according to Fujimura *et al.*[10]), and aqueous mobile phases. Upon evaluation a remarkable concentration-dependent behaviour was noticed, resulting in great difficulties when using this system in quantitative work.

In this paper we describe the results obtained with a commercially available β-cyclodextrin column. Some marked differences were seen compared to our column. Furthermore, we were able to determine the optical purity of enantiomers of cyclo-

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hexylphenylglycolic acid (CHPGA) between 99.5 and 100.0%. These data are essential for the interpretation of the results of pharmacological experiments with some derivatives of these acids, namely quaternary anticholinergic compounds. For this class of drugs it would otherwise be very difficult to determine their optical purity.

EXPERIMENTAL

Reagents

Acetonitrile, orthophosphoric acid (85%) and dipotassium hydrogenphosphate trihydrate were of analytical grade and obtained from E. Merck (Darmstadt, F.R.G.). Tropic acid (synthetic grade) was also obtained from E. Merck. Racemic CHPGA was a gift from Ciba-Geigy (Basle, Switzerland). Cyclohexylphenylacetic acid (CHPAA) and mandelic acid from Janssen (Beerse, Belgium) were used as received. Optical antipodes of CHPGA were obtained by crystallization from diastereomeric salts.

Apparatus

The experiments were performed with a Perkin-Elmer Series 10 HPLC pump, a Rheodyne 7125 sample injector, equipped with a 20-μl loop, a Spectra Physics 770 UV detector operating at 205 nm and/or an HP 8450A (Hewlett-Packard) multichannel UV–visible spectrophotometer and an Omniscribe Houston recorder. The diode-array spectrophotometer was equipped with a Model 178.32 QS quartz cell (Hellma) of 8 μl. Details of this detector as well as of the data handling are described elsewhere[11].

Differential scanning calorimetry was performed at the Laboratory of Pharmaceutical Technology and Dispensing.

Chromatographic conditions

The commercially available β-cyclodextrin column (I) (250 mm × 4.6 mm I.D.) was a gift from Serva (Heidelberg, F.R.G.). It was thermostatted by means of a water jacket, connected to a water-bath, kept at constant temperature as indicated. The mobile phase was 0.1 *M* potassium phosphate buffer (pH 4.3)–acetonitrile (60:40). The flow-rate was 1 ml/min. The preparation of our β-cyclodextrin stationary phase (column II) and the HPLC conditions have been reported[9].

RESULTS AND DISCUSSION

Comparison of the two β-cyclodextrin bonded stationary phases

Shortly after publication of the HPLC results obtained with our own synthesized β-cyclodextrin bonded stationary phase, we had the opportunity to evaluate a new commercially available β-cyclodextrin column.

We started with the mobile phase composition found to be optimal in our previous work, potassium phosphate buffer (pH 4.3, 0.1 *M*)–acetonitrile–methanol (62.5:31.5:6). CHPGA was separated into optical antipodes on column I with retention times of about 70 and 85 min, respectively, whereas the retention times were 10.5 and 13.5 min, respectively, on a column of the same dimensions packed with our home-made stationary phase (column II). The amount of organic modifier on

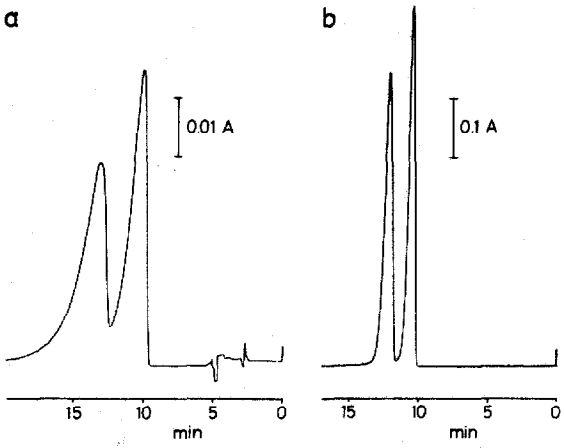

Fig. 1. Chromatograms of racemic CHPGA on different β-cyclodextrin columns. (a) Column II (250 mm × 4.6 mm I.D.). Mobile phase: phosphate buffer (pH 4.3, 0.1 *M*)–acetonitrile–methanol (62.5:31.5:6). sample: 2.7 μg *dl*-CHPGA in 20 μl. Temperature: 24.2°C. (b) Column I (250 mm × 4.6 mm I.D.). Mobile phase: acetonitrile–phosphate buffer (pH 4.3, 0.1 *M*) (60:40). Sample: 12 μg *dl*-CHPGA in 20 μl. Temperature: 25°C.

column I was then increased to 60% acetonitrile; higher percentages of acetonitrile caused problems due to precipitation of the buffer components. The retention times of the enantiomers were now reduced to 10.5 and 12.5 min, respectively. Symmetric peaks were observed with little tailing and a resolution of the optical antipodes of about 2. Fig. 1 depicts chromatograms of racemic CHPGA, obtained with both systems. It is clear that column I is preferable for this particular separation.

In addition, some other remarkable differences between the commercial and the home-made stationary phase were observed: (a) the elution order of the optical antipodes of CHPGA on the two columns is different; (b) two other aromatic carboxylic acids, cyclohexylphenylacetic acid and mandelic acid, were not separated into enantiomers on column I, although column II gave a good and a very small resolution, respectively. These phenomena can be explained by differences in the spacer arm and/or the synthesis procedure. The only known difference between the columns is the structure of the spacer arm. We used a $-(CH_2)_3NHCH_2CH_2NH-$ moiety for coupling the β-cyclodextrin to the silica gel, whereas Serva used a C_6 moiety[12]. In both cases, it is likely that the spacer arm couples predominantly to the primary hydroxyl groups of the cyclodextrin.

Retention of molecules on β-cyclodextrin bonded stationary phases is explained by inclusion, whereby guest molecules of appropriate size and shape can form strong inclusion complexes[13]. Other authors found that, in several cases, inclusion mechanisms alone could not explain the retention observed. Solubility or solvophobic type interactions may also be involved[14]. For aromatic carboxylic acids, anion-exchange mechanisms may play a rôle as well[15]. In the latter study the spacer arms used contained nitrogen atoms. Differences in the magnitude of these phenomena may explain the reversal of elution order of the optical antipodes of CHPGA on the two columns evaluated in this study: on column I, inclusion complex formation mainly determines the interaction, whereas on column II the retention mechanism will be the result of a combination of inclusion and anion exchange.

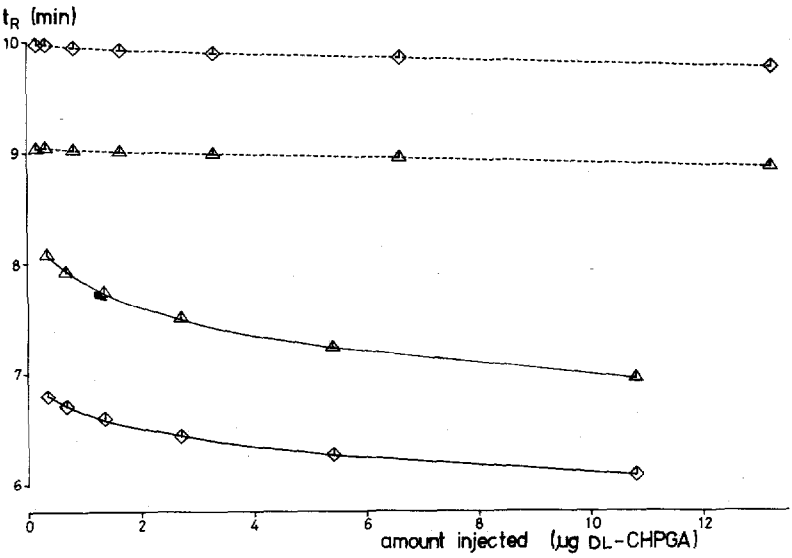

Fig. 2. Retention time, t_R, *versus* amount of CHPGA injected. ◇, *d*-CHPGA; △, *l*-CHPGA. ———, Column II, conditions as in Fig. 1a, except 47°C; – – –, column I, conditions as in Fig. 1b, except 40°C.

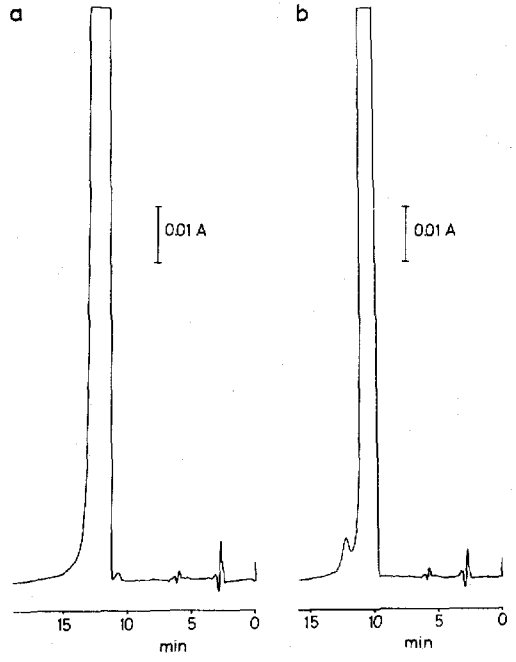

Fig. 3. Chromatograms of *l*- and *d*-CHPGA, which show a small amount of the other enantiomer as an impurity. Samples: (a) 15.6 µg *d*-CHPGA; (b) 12.4 µg *l*-CHPGA; other conditions as in Fig. 1b.

The tailing phenomenon, observed on column II, may also be due to interaction of the acids with –NH– functions of the spacer arm. This tailing was not seen with nitroanilines, which have almost no opportunity to interact with the amine function of the stationary phase.

Thus, various types of β-cyclodextrin columns may not necessarily exhibit the same separation characteristics. This is further supported by observations on cyclohexylphenylacetic acid, which could be separated into optical antipodes by Hinze *et al.*[13] with a separation factor, α, of 1.05, while we found a separation factor of 1.7 on column II[9] and could not separate this compound on column I using a mobile phase of 60% acetonitrile and 40% buffer (pH 4.3).

Besides severe tailing, there was another problem with column II. This column showed a strong concentration dependency of retention time, leading to great difficulties in quantitative work[9]. On column I the retention time was almost constant in the concentration range studied (Fig. 2). We therefore concluded that the latter could be used for determining the optical purity of enantiomers of CHPGA.

Determination of optical purity

On injections of large amounts of *d*- or *l*-CHPGA, small impurities were observed of the optical antipode (Fig. 3). In Fig. 4 a three-dimensional chromatogram of the levorotary antipode is presented. The small impurity is seen on the tail, *i.e.*, at the front in this figure, of the major antipode. From such figures we also concluded that no other impurities were eluted from the column, at least in the wavelength range from 200 to 350 nm.

Calibration curves for the enantiomers were constructed in the range of the estimated impurity. Quantitations were done by integration of the peak area, as well

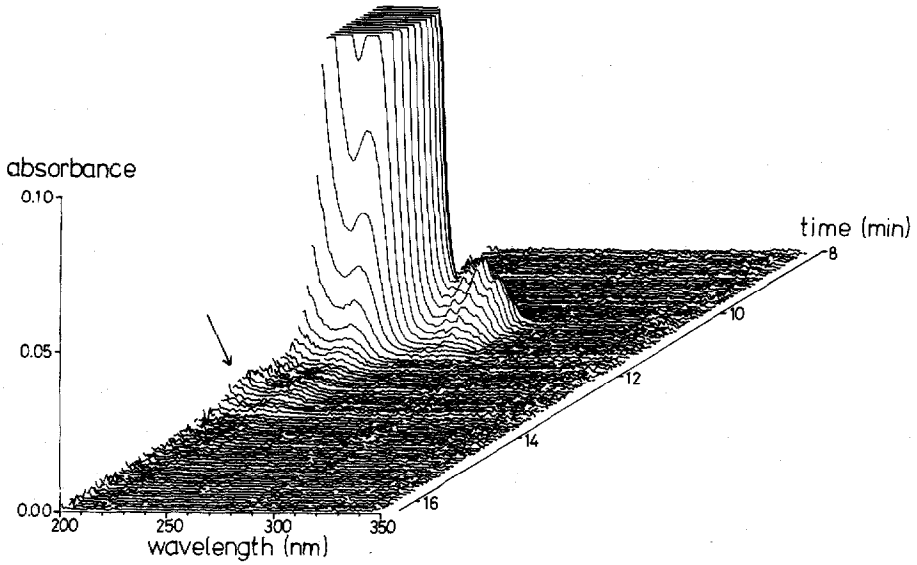

Fig. 4. Three-dimensional chromatogram of *l*-CHPGA (reversed time axis). Conditions as in Fig. 3b; detection with diode-array. The contaminant *d*-CHPGA is indicated by an arrow.

TABLE I

OPTICAL PURITY (%) OF CHPGA ENANTIOMERS DETERMINED BY THREE METHODS

DSC = Differential scanning calorimetry. Chromatographic conditions as in Fig. 3.

	DSC	*Chromatography*	
		Area	*Peak height*
l-CHPGA	99.57	99.76	99.65
d-CHPGA	99.90	99.94	99.92

as by manual measurement of the peak height. In both cases the correlation coefficient was 0.9999.

Purity was also determined by differential scanning calorimetry (DSC), based on the Van't Hoff equation[16]. The results are presented in Table I. Strictly, the DSC method determines both the optical and chemical purity and it should be used only for the determination of optical purity if it is shown that the compounds under investigation are chemically pure. This was assumed to be the case as indicated above.

We conclude that this chromatographic system meets our demands for the determination of the optical purity of the enantiomers of CHPGA.

ACKNOWLEDGEMENTS

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