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Liquid and high-pressure carbon dioxide chromatography of β-blockers

Resolution of the enantiomers of nadolol[a]

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ABSTRACT

The enantiomers of six chiral aryloxypropanolamine β-blockers were separated by high-performance liquid chromatography on a column of modified cellulose immobilized onto silica (Chiralcel OD), using mixtures of heptane or hexane with a polar modifier. The quality of the separations was improved and retention times were reduced by eluting under subcritical conditions with carbon dioxide containing 20% of methanol. Nadolol, which has two chiral centres, gave three peaks instead of four on Chiralcel OD. A separation of the four isomers was obtained on an α_1-acid glycoprotein column eluted with an aqueous buffer containing octanoate ions. A direct separation of the two diastereomers could not be obtained with achiral stationary phases, but the derivative formed by the base-catalysed reaction of nadolol with isopropyl isocyanate was well resolved on a column of amino-bonded silica eluted with carbon dioxide–methanol. This paper demonstrates the advantages of high-pressure carbon dioxide chromatography for the analysis of stereoisomers.

INTRODUCTION

The chirality of drugs is an important issue from the pharmacological, pharmacokinetic, toxicological, and regulatory points of view [1,2]. Despite the lack of a predictive mechanistic theory, direct chromatographic chiral separations have been described for compounds having numerous different functions [3]. The currently available chiral stationary phases can be classified according to their chemical properties and, in general, each type of phase is suitable for compounds having a limited range of functionalities [4,5]. The β-adrenoceptor antagonists (β-blockers) are a clinically important class of drugs used in the treatment of cardiovascular disorders and of ocular hypertension. They have in common a substituted aryloxypropanolamine function of which the hydroxy-substituted carbon is chiral (see

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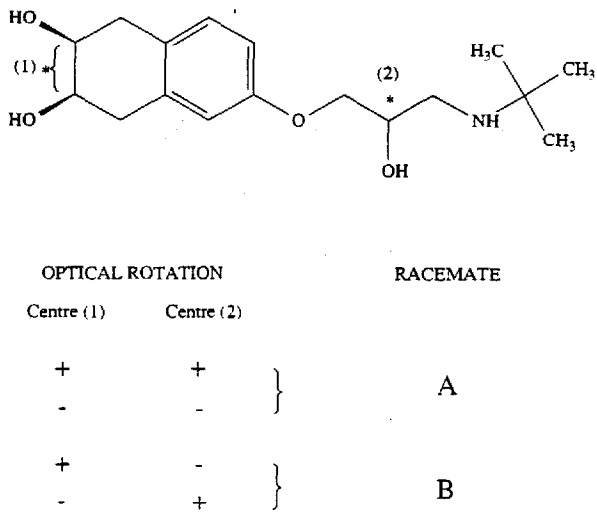


Fig. 1. Structure of nadolol.

nadolol, Fig. 1). Despite this structural similarity between members of the series, the racemic compounds display a wide variety of pharmacological properties.

Mode-of-action studies of the β-blockers reveal marked enantioselectivity (*e.g.*, refs. 6 and 7), and therefore the pharmacological profiles of racemic β-blockers are composites of the profiles of the enantiomers. As both enantiomers usually have some relevant activity, an optically pure β-blocker is not necessarily a "better" product than the racemate; it is rarely possible to describe a racemate as a mixture of an active substance and "isomeric ballast" [8]. Nevertheless, the synthesis and analysis of optically pure β-blockers represents a field of much commercial importance.

Chiral chromatographic separations, mostly with stationary phases of modified cellulose grafted onto silica (Chiralcel series), which are suitable for pharmaceutical analysis and pharmacokinetic studies, have been described for all of the important aryloxypropylamines [4]. Chiralcel columns are used in the normal-phase mode and they are usually eluted with a mixture of hexane and polar modifiers such as *n*-propanol [4]. Normal-phase separations are greatly improved in terms of speed and resolution by eluting with mixtures of carbon dioxide and methanol, in which solutes have relatively favourable diffusion coefficients [9,10]. This technique is generally referred to as supercritical fluid chromatography (SFC). However, as the state of the fluid used in this present study is not always supercritical, we shall use the abbreviation in quotation marks. "SFC" is now used routinely in many laboratories and it is perhaps time to adopt a convenient but more theoretically sound vocabulary. The essential feature of "SFC" is that a fluid that would be gaseous at pressures near atmospheric is compressed to a density at which it has significant solvating power. The principal advantages of "SFC" over conventional liquid chromatography are that diffusion coefficients are greater, and that the solvating power can be varied by means of pressure-induced density changes. Bearing in mind that the main constituent of the eluent is usually CO_2, we suggest that an expression such as "high-pressure carbon

dioxide chromatography (HPCDC)" would be more generally appropriate than "SFC" whenever the solvating power of the CO_2 plays a significant role in the chromatographic mechanism. This type of expression correctly applies to all applications of this particular eluent at pressures higher than those generally used in gas chromatography [11]. We have been unable to devise an expression that would cover all "SFC" mobile phases.

Previously, we described chiral separations of six β-blockers by high-performance liquid chromatography (HPLC) on a Chiralcel OD column [4]. In this paper, we evaluate the improvements that can be obtained by using "SFC" techniques with particular emphasis on the resolution of the isomers of nadolol. Among the clinically important β-blocking drugs, nadolol presents a particular chromatographic challenge as it possesses two chemically different chiral sites, the second being a *cis*-1,2-dihydroxy function (Fig. 1). The commercial drug substance is a mixture of approximately equal proportions of the two racemic diastereomers, the $+-/-+$ (*RS*/*SR*) mixture being designated racemate A and the $++/--$ (*RR*/*SS*) mixture racemate B [12]. The diastereomers are separable by fractional crystallization, but they are surprisingly difficult to separate chromatographically. One report mentions unsuccessful trials in which 202 different solvent systems were tested on 14 different thin-layer chromatographic supports [12]. A method using reversed-phase chromatography was reported in 1981 [13], but we were unable to reproduce this result using numerous currently available column packings. In 1984, an incomplete ion-exchange separation was described [14]; the analysis time was 20 min. In the absence of a robust chromatographic method for separating the diastereomers of underivatized nadolol, the official method for analysing the drug substance [15] is infrared spectroscopy, a relatively insensitive and inaccurate technique. In this paper, we show that the reaction of nadolol with isopropyl isocyanate [16,17] in the presence of a base yields a derivative whose diastereomers are well separated by normal-phase chromatography on a column of amino-bonded silica.

Nadolol gave at most three partially resolved peaks when analysed on the columns we tested during the study of chiral separations by HPLC on Chiralcel OD cited above [4]. In 1986, Schill *et al.* [18] described the chiral separations of the individual diastereomers on an α_1-acid glycoprotein (α_1-AGP) stationary phase. However, one member of each enantiomeric pair had the same retention time, so that a mixture of the diastereomers would, as on the Chiralcel OD column, have given three peaks instead of four. In addition, the original α_1-AGP columns were not ideally suited to pharmaceutical analysis; their loading capacity was limited, retention times were long and the peaks were generally broad. These factors impaired the detection of trace levels of the unwanted enantiomer. We now report that these difficulties are less severe with a "second generation" α_1-AGP column [19], and that, by carefully selecting the mobile phase, a complete separation of the four isomers of nadolol can be obtained.

EXPERIMENTAL

Reagents and chemicals

A racemic mixture containing approximately equal proportions of the diastereomers of nadolol was kindly supplied by Squibb. Isopropyl isocyanate (>99%) was purchased from Fluka (Buchs, Switzerland). Liquid carbon dioxide, quality N 45 (<7

TABLE I

CHIRAL AND NON-CHIRAL COLUMNS TESTED FOR THE CHROMATOGRAPHY OF β-BLOCKERS

Column	Supplier	Dimensions (mm)	Comments	No. of peaks[a]
Suplex pKb-100	Supelco	150 × 4.6	Reversed-phase	1
Erbasil 5 C_{18}/H	Carlo Erba	150 × 4.6	Reversed-phase	1
Zorbax Phenyl	DuPont	150 × 4.6	Reversed-phase	1
Spherisorb Mixed-Mode	Phase Separations	150 × 4.6	Reversed-phase, normal-phase (HPLC, SFC)	1
Spherisorb Phenyl	Phase Separations	150 × 4.6	Reversed-phase; chiral ion-pair with ZGP (HPLC, SFC)	1
Asahipak ODP-50	Asahi	150 × 4.6	Reversed-phase	1
Spherisorb S5 CN	Phase Separations	150 × 4.6	Normal-phase; chiral ion-pair with ZGP (HPLC, SFC)	1
Partisil 5	Whatman	150 × 4.6	Normal-phase	1
Zorbax BP-SIL	DuPont	150 × 4.6	Normal-phase	1
Spherisorb NH_2	Phase Separations	150 × 4.6	Normal-phase, derivatization with isopropyl isocyanate (HPLC)	1
Spherisorb NH_2	Phase Separations	150 × 4.6	Normal-phase, derivatization with isopropyl isocyanate (SFC)	2
LiChrospher Diol	Merck	150 × 4.6	Normal-phase	1
Chiral Pro-Cu-Si 100 Polyol	Serva	250 × 4.6	Ligand exchange	1
Cyclobond I (10 μm)	Astec	250 × 4.6	Reversed-phase, normal-phase (HPLC, SFC)	1
Chiralcel OD (10 μm)	Daicel	250 × 4.6	Normal-phase (HPLC, SFC)	3
Chiralcel OC (10 μm)	Daicel	250 × 4.6	Normal-phase (HPLC, SFC)	2
Chiralcel OG (10 μm)	Daicel	250 × 4.6	Normal-phase (HPLC, SFC)	2
Chiral-AGP	Baker	100 × 4.0	"Affinity chromatography" with ion-pairing (HPLC)	4
Pirkle covalent L-phenylglycine	Regis	250 × 4.6	Normal-phase, derivatization with isopropyl isocyanate (SFC)	2
Supelcosil-LC-(R)-urea	Supelco	250 × 4.6	Normal-phase, derivatization with isopropyl isocyanate (SFC)	2

[a] Number of peaks obtained for the racemic mixture of the two diastereomers of nadolol (four isomers).

ppm of water) was purchased from Alphagaz (Bois-d'Arcy, France). Other reagents and solvents were obtained from commercial sources.

Columns

The chiral and non-chiral columns used are listed in Table I.

Apparatus ("SFC" and HPLC)

Chromatography was performed using equipment manufactured by Jasco (Tokyo, Japan; supplied by Prolabo, Paris, France), except where indicated otherwise. The two pumps (Model 880-PU) are connected for gradient elution by means of a Model 801-SC controller and Model MX-50 dynamic high-pressure mixing unit. The head of one of the pumps is cooled to $-2°C$ when CO_2 is used. The mixing unit is connected to a standard injector (Model 7125; Rheodyne, Cotati, CA, U.S.A.) via a pressure relief valve (Rheodyne Model 7037). The column is mounted in a thermostatically controlled oven. The UV detector (Model 875-UV) is fitted with the standard high-pressure cell (4 μl; 5-mm path length). When CO_2 is used, the eluent is discharged via an automatic back-pressure regulator (Model 880-81). Chromatograms were recorded using an electronic integrator (Model C-R3A; Shimadzu, Kyoto, Japan; supplied by Roucaire, Vélizy-Villacoublay, France).

Chromatography of underivatized nadolol

Nadolol was dissolved in appropriate solvents at a concentration of 0.1% and 20 μl were injected. For each column, optimization of the "SFC" separation was attempted by varying the temperature between 20 and 45°C and the pressure between 100 and 280 bar and by varying the nature and concentration of the polar modifier.

Several hundred micrograms of nadolol were fractionated by repetitive injections onto the Chiralcel OD column, under the optimized conditions. The fractions corresponding to each of the three peaks were separately pooled and the solvent was evaporated in a vacuum centrifuge. The optical activities of methanolic solutions of the fractions were measured using a Jasco Model DIP-370 polarimeter (sodium D-line). Subsequently, the fraction corresponding to the first-eluted peak was re-evaporated and the residue was derivatized as described below.

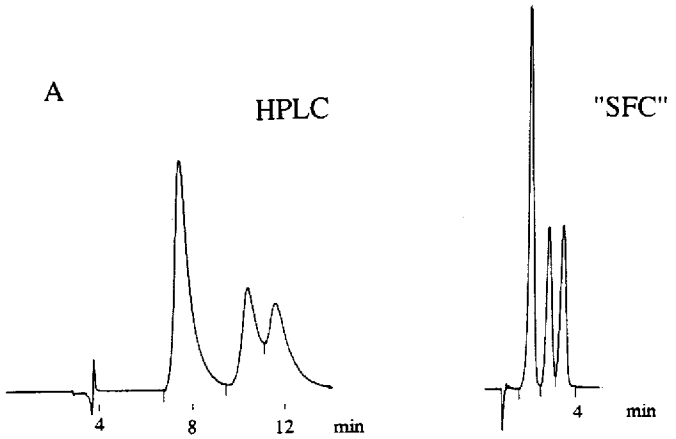
Derivatized nadolol

A suspension of nadolol (10 mg) in dichloromethane (350 μl), triethylamine (5 μl) and isopropyl isocyanate (150 μl) was prepared in a small conical glass tube fitted with a B-10 ground-glass stopper. The tip of the tube was heated at 60°C for 1 h, after which the solvent and excess of reagents were evaporated under a stream of nitrogen at room temperature. The pale brown residue was dissolved in a few drops of methanol, which was evaporated, and the derivative was taken up in methanol.

Exact experimental conditions for the separations that were studied in detail are given in the legends to the figures.

RESULTS AND DISCUSSION

The chiral and non-chiral columns used are listed in Table I, together with an indication of the mode of elution and the results obtained for nadolol. Chiralcel OD



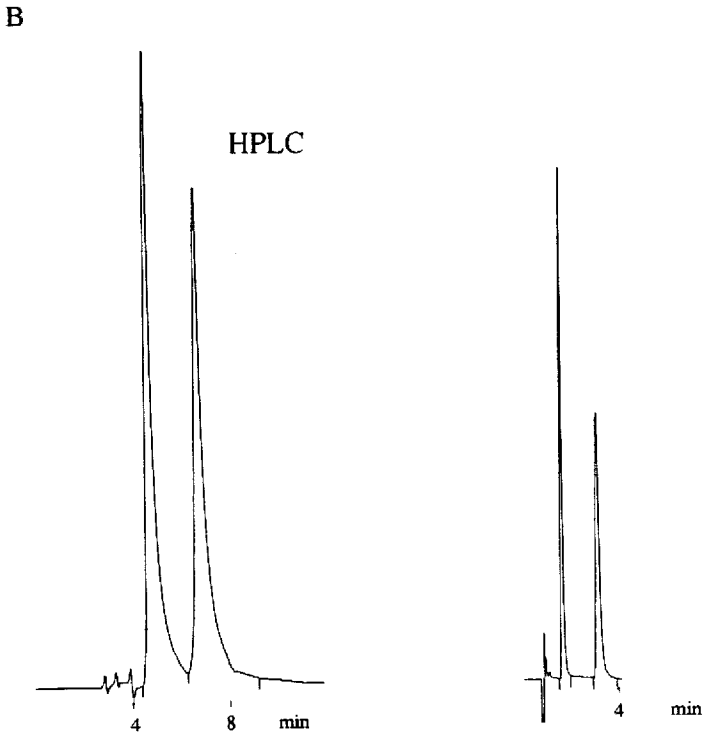


Fig. 2.

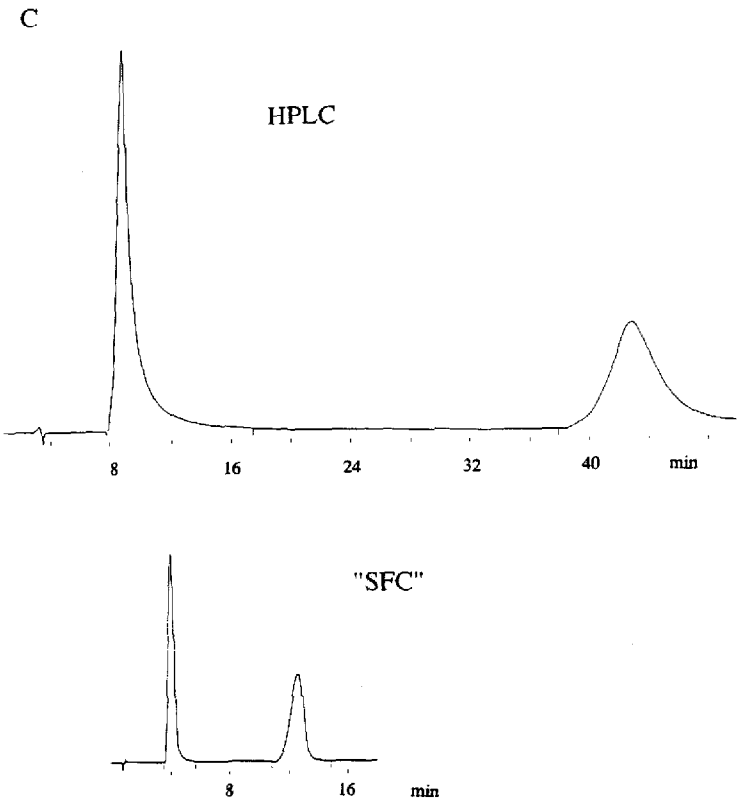


Fig. 2.

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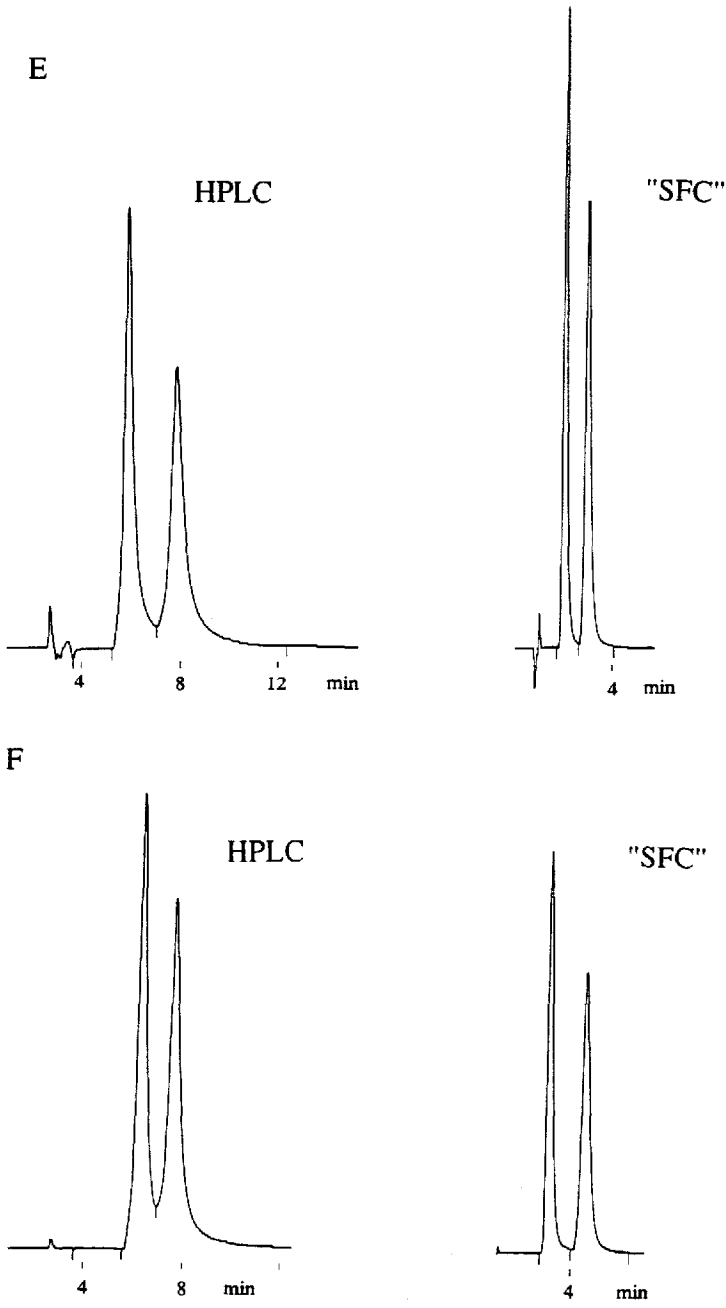

Fig. 2. Separations of β-blockers on Chiralcel OD (10 μm) column (250 × 4.6 mm I.D.) by HPLC and "SFC", with UV detection at 254 nm. (A) Nadolol; the mobile phase for HPLC was hexane–ethanol–2-propanol (80:15:5, v/v/v), temperature 22°C, flow-rate 1.0 ml/min; the mobile phase for "SFC" was CO_2–methanol (80:20, v/v), temperature 35°C, pressure 200 bar, flow-rate 4.0 ml/min. (B) Betaxolol hydrochloride; the HPLC mobile phase was hexane–2-propanol (83:1,7 v/v); the other chromatographic conditions were the same as in A. (C) Pindolol; the chromatographic conditions were the same as in A. (D) Metoprolol; the chromatographic conditions were the same as in A. (E) Cicloprolol hydrochloride; the chromatographic conditions were the same as in A. (F) Propranolol; the chromatographic conditions were the same as in A.

proved to be generally useful for all the β-blockers tested and, because this stationary phase functions in the normal-phase mode, we investigated the improvements that could be obtained by the use of "SFC".

Enantiomeric separations of β-blockers on Chiralcel OD: comparison between HPLC and "SFC"

Chromatograms of six β-blockers were obtained using the same Chiralcel OD column under standard HPLC conditions (elution with mixtures of hexane or heptane and propanol) and under "SFC" conditions (CO_2–methanol). The results, presented in Fig. 2, clearly indicate that, although the separations obtained by the two techniques are qualitatively similar, the "SFC" technique gives superior efficiencies and shorter retention times. The "SFC" traces were obtained under conditions that had been optimized, with a view to obtaining adequate separations with short retention times, by varying the temperature, pressure and flow-rate, as well as the concentration of methanol.

As expected for normal-phase chromatography, increasing the concentration of methanol led to decreased retention indices and a slight loss in efficiency. At the methanol concentration (20%) and temperature (35°C) finally chosen, it is most likely that the eluent is far below its critical temperature [20], although there appears to be no general agreement on the appropriate equations for calculating the critical parameters of mixtures [21]. The effects of varying, at a constant mass flow-rate, the temperature and the pressure at the column outlet are shown in Fig. 3 for betaxolol. Most interesting is a marked decrease in capacity factor (k') with increasing pressure, typical of separations usually described as "supercritical", although the pressure dependence was about the same at 20°C as at 45°C. The simplest explanation for this observation would be that the solvent mixture is more compressible than conventional solvents.

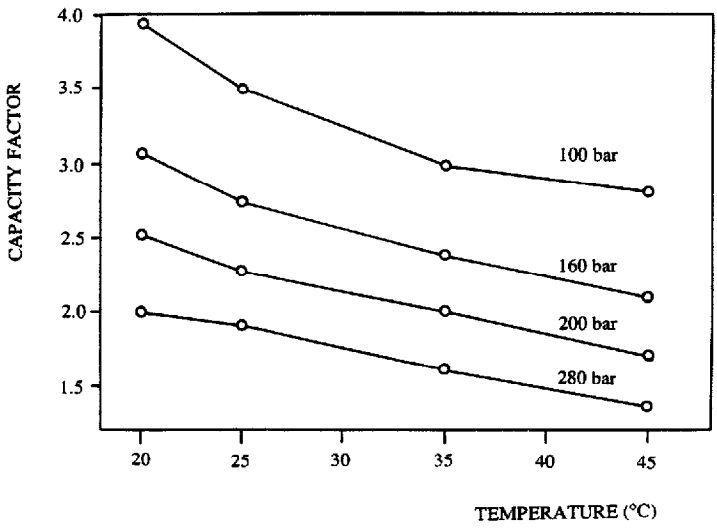

Fig. 3. Variation of k'_2 of betaxolol in "SFC" as a function of pressure and temperature. The chromatographic conditions were the same as in Fig. 2B.

The retention index decreased with increasing temperature, an effect that is always observed in liquid chromatography. For practical purposes, for the separations described here, the improvement afforded by the use of "SFC" can be attributed to the more favourable chromatographic properties (diffusion coefficients and viscosity) of CO_2–methanol compared with hexane [9,10]. The advantages of mobile phases that would be gaseous at atmospheric pressure extend to temperatures well below the

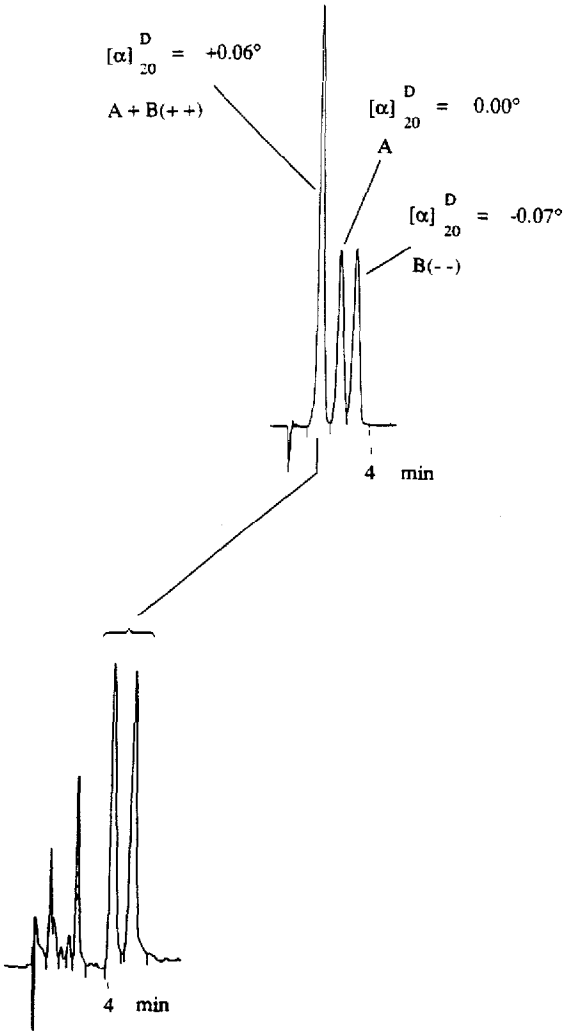

Fig. 4. Characterization of the stereoisomers of nadolol. Upper panel: optical activities and postulated configurations of the three peaks separated by "SFC" on a Chiralcel OD column. The chromatographic conditions were the same as in Fig. 2A. Lower panel: "SFC" separation of isopropyl isocyanate derivative of the first peak on the upper panel, collected and reanalysed by "SFC" with a Spherisorb-NH_2 (5 μm) column (100 × 4.6 mm I.D.); mobile phase CO_2–methanol (95:5, v/v), temperature 25°C, pressure 200 bar, flow-rate 3.0 ml/min.

critical point; at 20°C, the viscosity of liquid CO_2 is 0.071 cP, whereas that of hexane is 0.326 cP [22].

Nadolol: separations on Chiralcel OD

The Chiralcel OD column used gave three peaks for nadolol (Figs. 2A and 5A), whereas during an earlier study with another column of the same type we could obtain only two peaks. It is difficult to explain this batch-to-batch variation. Attempts to improve the separation further were unsuccessful. The first of the three peaks had twice the area of the other two, indicating that the two less intense peaks represent two of the four optically pure isomers that form the mixture (Figs. 4 and 5). As the separate diastereomers were not available to us, we collected fractions corresponding to the

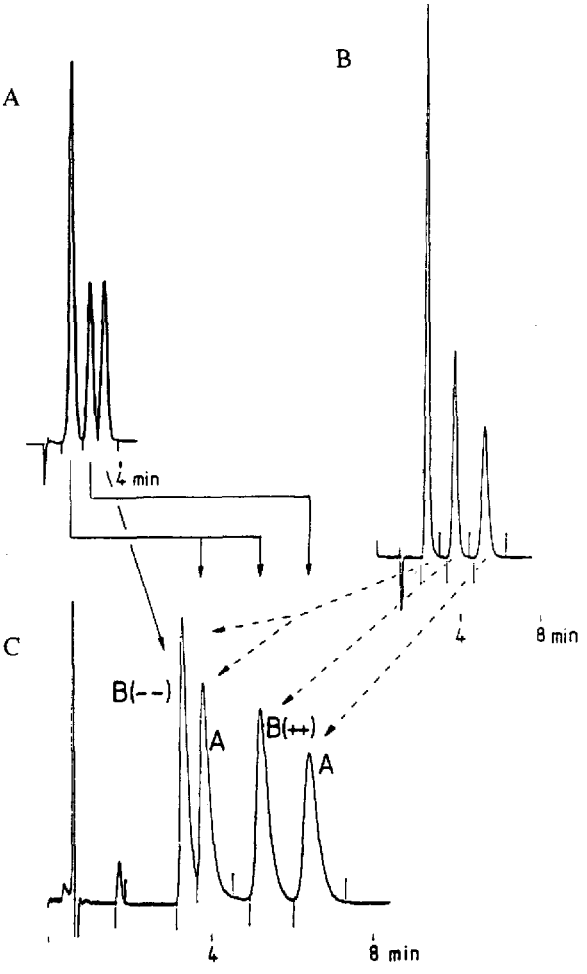

Fig. 5. Separations of the isomers of nadolol. (A) Chiralcel OD as in Fig. 2A. (B) α_1-AGP; the mobile phase was KH_2PO_4 (0.025 *M*) and tetrabutylammonium bromide (0.1%), adjusted to pH 7.5 with NH_3, temperature 22°C, flow-rate 0.9 ml/min. (C) α_1-AGP; the chromatographic conditions were as in (B), except that the mobile phase contained 0.05% octanoic acid instead of tetrabutylammonium bromide.

three peaks. These fractions gave optical activities of +0.06°, 0.00° and −0.07° respectively; although the measurements are close to the limit of detection of the polarimeter, it is clear that the activities of the second and third peaks are not of opposite sign. Therefore, they represent one enantiomer of each of the diastereomeric pairs. The other two enantiomers, which form the first peak, are also diastereomerically related. Further, since the enantiomers of the diastereomer designated racemate B [12] have the configurations (+ +) and (− −), the third peak, which has a larger absolute value of α than the second (Fig. 5A), can be assigned as the isomer B(− −). The second peak therefore represents one of the enantiomers of racemate A.

Nadolol: chiral separations on α_1-AGP

As the isomers of nadolol were not fully resolved on the Chiralcel columns, we reinvestigated the use of a new "second generation" α_1-AGP column. When eluted with a phosphate buffer containing tetrabutylammonium ion [18], three peaks were obtained for nadolol (Fig. 5B). The separation was qualitatively the same as that described by Schill *et al.* [18], but the efficiency, resolution and analysis time were all more favourable. In the absence of tetrabutylammonium ion, retention times were longer and the three peaks were broader and less well separated (not shown). The effects of different mobile phase additives were tested, and the results obtained with a buffer containing 0.05% of octanoic acid are shown in Fig. 5C. This chromatogram

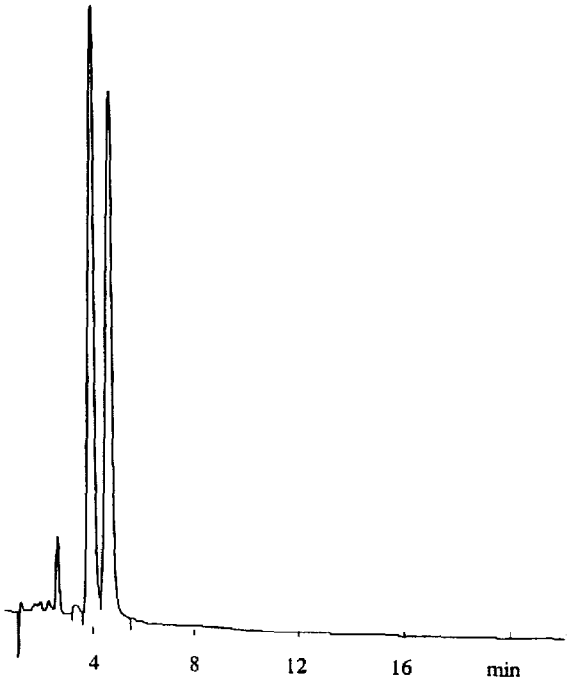

Fig. 6. "SFC" separation of the diastereomers of nadolol derivatised with isopropyl isocyanate in the presence of triethylamine, on a Spherisorb-NH_2 column. The chromatographic conditions were the same as in Fig. 4 (lower panel).

has four peaks of equal areas, separated almost to the baseline. With lower concentrations of octanoic acid, the first two peaks began to coalesce; at higher concentrations the second peak overlapped the third. Presumably, the octanoate modifies the relative retention times by forming ion pairs with the solutes, as both the additive and the stationary phase are negatively charged. The peaks in Fig. 4 were identified (1) by injecting the fractions (of known relative optical activities) from the Chiralcel OD column (Fig. 5A) onto the α_1-AGP column and (2) from the relative retention times of the A and B diastereomers given for α_1-AGP by Schill *et al.* [18]. There was insufficient information to distinguish the enantiomers of diastereomer A.

Nadolol: separation of the diastereomers on non-chiral columns

Because of doubts about the long-term availability and reproducibility of chiral columns, an achiral chromatographic method for separating the diastereomers would be of value as a replacement for the infrared spectroscopic method currently used for the quality control of nadolol. We attempted without success to separate the diastereomers of underivatized nadolol with the columns and eluents listed in Table I. Bearing in mind the unsuccessful efforts of other workers (see Introduction), there appears to be little further scope for this approach. We therefore turned to the use of a derivative. Isopropyl isocyanate was chosen as derivatizing reagent, because it reacts

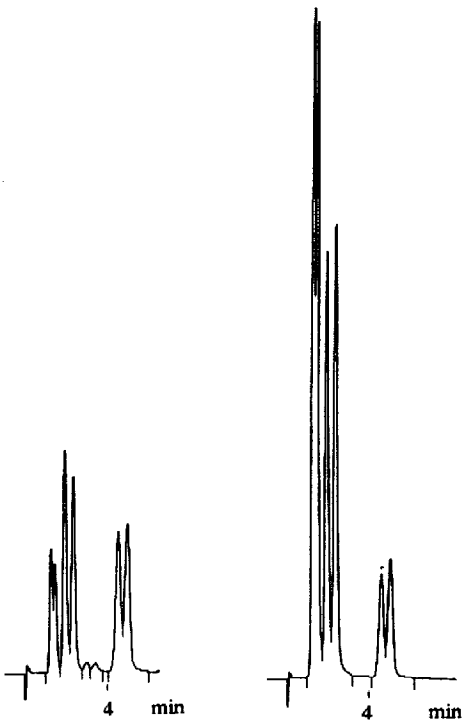

Fig. 7. Separations of the diastereomers of nadolol derivatised with isopropyl isocyanate in the absence of triethylamine (two preparations). The column was the same as in Fig. 4 (lower panel); mobile phase CO_2–methanol (90:10, v/v), temperature 25°C, pressure 200 bar, flow-rate 4.0 ml/min.

with most common functional groups that possess an active hydrogen to give neutral products, and because several cases of enhanced chromatographic stereoselectivity have been described by König's group [16,17]. Following reaction under the conditions described under Experimental, the racemic mixture of nadolol diastereomers gave two well resolved peaks of equal areas when chromatographed using "SFC" conditions on a non-chiral column (amino-bonded silica; Fig. 6). When the reaction was carried out in the absence of triethylamine, the chromatogram showed several pairs of peaks, presumably representing different incompletely derivatized products (Fig. 7), for each of which the diastereomeric pair was separated. As, when triethylamine was used, the products formed represent the least retained pair of derivatives, and as no other significant peaks appeared on the chromatogram, it can be assumed that the catalysed reaction was virtually quantitative.

Surprisingly, apart from the Spherisorb-NH_2 column, none of non-chiral columns listed in Table I separated the diastereomers of the isopropyl isocyanate derivative.

Isopropyl isocyanate derivative of nadolol: separation of the isomers on chiral columns

As the separation of the diastereomers of nadolol on an achiral column was facilitated by derivative formation, it was hoped that a chiral column might separate all four isomers of the derivative. All the chiral stationary phases (except α_1-AGP) listed in Table I were tested under "SFC" conditions, but the results were disappointing. The Pirkle-type covalently-bonded L-phenylglycine and the Supelcosil-LC(R)–urea columns gave two but not four peaks, and the other columns gave only one.

CONCLUSION

As the retention mechanism involved in chiral chromatographic separations on modified celluloses is essentially normal-phase, this is a field in which "SFC" can be used to advantage. All of the common β-blockers except nadolol are fully resolved by this approach. The direct chiral analysis of the four isomers of nadolol can now be carried out on the α_1-AGP stationary phase. Alternatively, Chiralcel OD may be used, provided that the diastereomeric ratio is also determined by chromatography of a derivative. The choice between these two methods may depend, ultimately, on the reproducibility of the commercially available columns.

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