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Chiral separations of β -blocking drug substances using chiral stationary phases

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Abstract

Three chiral stationary phases were evaluated for their usefulness in determinations of optical purity and separations of β -blocking drug substances. The phases were a modified cellulose phase (Chiralcel OD), an immobilized protein (AGP) and a β -cyclodextrin phase (Cyclobond I). These phases were evaluated concerning their practical analytical use for the determination of the enantiomeric purity of non-racemic β -blocking substances. The best phase proved to be Chiralcel OD, which provided excellent separations of almost all β -blocking substances tested and rugged determinations performed with satisfactorily low detection limits. Similar results could be obtained with AGP, but this did not seem to be sufficiently rugged for routine analysis. The β -cyclodextrin phase resulted in poor chiral separations of most β -blocking substances and the usefulness of this phase for analytical purposes seems limited.

1. Introduction

It is well known that the different enantiomers of most optically active drug substances can possess different biological activities. This is also a concern of the regulatory authorities if an optically active compound is to be registered as a drug substance [1,2]. If a compound that is intended to be marketed as a drug is one enantiomer, the regulatory authorities will regard the presence of the other enantiomer, in the raw material or in the finished product, following the same principles as for any other chemical impurity. Hence the need for methods for the determination of one enantiomer in the other

has increased. The classical method for the determination of optical purity, also in pharmacopoeial monographs of drug substances, is optical rotation. However, this method is neither very accurate nor precise, and it will be difficult to control the content of enantiomeric impurity at low levels. In recent years, HPLC techniques that are capable of separating and determining enantiomers have been greatly improved. These techniques offer direct and specific determinations of enantiomeric purity. The particular interest in our laboratory has been to develop rugged and precise methods for use for standardization purposes, e.g., as part of testing procedures of drug substances as described in pharmacopoeial monographs. This investigation forms part of a series in which various ap-

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proaches concerning the possibilities of standardized HPLC methods for testing the optical purity of β -blocking drug substances are being evaluated [3].

β -Blocking drug substances most often exhibit a chiral structure and are mostly used as racemic mixtures with the *S*-form being responsible for the desired biological effect. An exception is sotalol, but this is not due to an opposite steric configuration at the chiral centre, but is caused by the ligand nomenclature rules. However, some β -blocking substances are marketed as single enantiomers. In Denmark, eighteen β -blocking drug substances are on the market, three of which are marketed as the pure *S*-form, timolol, bunolol and penbutolol. The remaining fifteen are marketed as the racemic mixture.

Three possible approaches to the separation of chiral substances by HPLC exist: (1) derivatisation of the analyte with a chiral reagent and formation of a diastereomer that can be separated by conventional achiral chromatography; (2) addition of chiral additives to the mobile phase and the use of an achiral stationary phase; and (3) the use of chiral stationary phases.

In our laboratory, the usefulness of determining optical purity by normal-phase HPLC following derivatization with (–)-camphanic acid chloride, (*S*)-(–)-1-phenylethyl isocyanate or 2,3,4,6-tetra-*O*-acetyl- β -*D*-glucopyranosyl isothiocyanate has been investigated previously [3]. This reference gives a thorough review and discussion of the literature on chiral separations of β -blockers with the use of derivatization.

Chiral separations performed by the addition of agents to the eluent have been studied with (+)-10-camphorsulphonate [4] and *N*-benzoxycarbonylglycyl-L-proline [5,6].

The simplest way to separate enantiomers is by direct injection of a sample on to a chiral stationary phase. Several chiral stationary phases have been used for the separation of enantiomers of β -blocking drug substances. Chiral phases consisting of immobilized proteins such as bovine serum albumin (BSA) [7], α -acid glycoprotein (AGP) [8–13], cellulohydrolase [13–18], ovomucoid [13,19–21] and various modifications of the ovomucoid protein [22,23] have

been used to separate enantiomers of β -blockers. The use of non-protein-based phases has also been reported: β -cyclodextrin in both the normal- and reversed-phase modes [13,24,25], Pirkle-type columns [26–29] and cellulose tris(3,5-dimethylphenylcarbamate) [13,30–46].

However, most work seems to have been done only to investigate the separating power and the separation mechanisms of the chiral stationary phases. So far, no work has been done to investigate the suitability of the column materials to determine enantiomeric purity in β -blocking substances at the level where other chemical impurities can be determined by the use of non-chiral analytical HPLC. The aim of this work was to evaluate a number of selected columns for this purpose. All β -blocking drug substances currently on sale in Denmark were included in the investigations in order to evaluate the stationary phases in the best possible way.

From the literature, the most suitable column seemed to be cellulose tris(3,5-dimethylphenylcarbamate) (Chiralcel OD), so such a column was chosen for further investigations. The use of protein-based columns for the separation of enantiomers of β -blocking substances is widespread. Of these protein based phases, α -acid glycoprotein (AGP) is one of the best known and seemed to be the best column to achieve our goal. Most of the Pirkle-type phases seemed less suitable, not because of all low separation power, but because derivatization of the substances is required prior to injection of the sample, thus diminishing the advantage of using a chiral stationary phase compared with a chiral derivatization procedure. One commercially available Pirkle-type phase has been specially designed for the separation of β -blockers without derivatization. However, preliminary work with this phase in our laboratory revealed some unwanted peak-splitting problems due to the temperature dependence of the separations. Further, this phase has been poorly investigated and only for a few substances [29]. Further work to investigate these problems is in progress. Cyclodextrins as stationary phase for the separation of β -blockers are not very useful when operated in the reversed-phase mode. However,

some work has been done with this phase using polar organic solvents as mobile phase, and this approach seems to provide a higher separation power towards β -blockers [25]. Therefore, a cyclodextrin stationary phase was also included in our investigations.

2. Experimental

2.1. Chemicals

Table 1 lists the compounds investigated and the companies from which they were obtained. The pure enantiomeric forms of timolol were supplied by Merck, Sharp & Dohme (Rahway, NJ, USA) and the pure forms of bunolol by Allergan (Irvine, CA, USA). Molecular structures are shown in Fig. 1. All other drug sub-

stances were of pharmacopoeial grade. Diethylamine was of analytical-reagent grade from Fluka (Buchs, Switzerland), water was purified by ion exchange prior to use and all other solvents were of analytical-reagent grade from Merck (Darmstadt, Germany).

2.2. Apparatus

The chromatographic systems consisted of a Kontron T 414 pump, an LDC SpectroMonitor variable-wavelength detector and a Rheodyne Model 7120 injection valve, an L 6200 pump, an L 4250 UV-Vis detector, an L 5025 column thermostat and an AS 2000A autosampler, all from Merck-Hitachi and a Kontron LC 410 pump with a Kontron Uvikon 735 LC detector. All chromatograms were recorded on a Kipp & Zonen BD-8 recorder. Data were collected on a Hewlett-Packard Model 3359A laboratory data system.

2.3. Columns

The cellulose tris(3,5-dimethylphenylcarbamate) phase was Chiralcel OD (25 cm \times 4.6 mm I.D., 10 μ m) from Daicel Chemical Industries (Tokyo, Japan). The α -acid glycoprotein phase was a Chiral-AGP column (100 \times 4 mm I.D., 5 μ m), used in connection with a guard column (10 \times 3 mm I.D.) packed with a similar phase, from ChromTech (Norsborg, Sweden). The β -cyclodextrin column was Cyclobond I (25 cm \times 4.6 mm I.D.) from ASTEC (Whippany, NJ, USA).

The chromatographic procedures used are given in the discussion section for the various stationary phases.

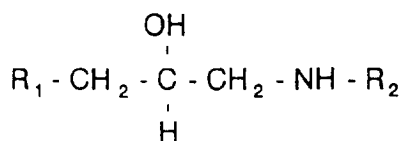
3. Results and discussion

3.1. Cellulose tris(3,5-dimethylphenylcarbamate) (Chiralcel OD)

Table 2 gives the chromatographic results obtained using the cellulose tris(3,5-dimethyl-

Table 1
 β -Blocking drug substances investigated and their sources

Compound	Company
Acebutolol	Rhône-Poulenc (Essex, UK)
Alprenolol	Hässle (Möln dal, Sweden)
Atenolol	ICI (Cheshire, UK)
Atenolol	Benzon Pharma (Hvidovre, Denmark)
Betaxolol	MEDA (Herlev, Denmark)
Betaxolol	Searle (Möln dal, Sweden)
Bevantolol	Parke-Davis (Ann Arbor, MI, USA)
Bevantolol	Benzon Pharma
Bisoprolol	Merck (Darmstadt, Germany)
Bunolol	Allergan (Irvine, CA, USA)
Carazolol	Upjohn (West Sussex, UK)
Carteolol	Ercopharm (Vedbaek, Denmark)
Metipranolol	Ciba-Geigy (Basle, Switzerland)
Metoprolol	Hässle
Oxprenolol	Ciba-Geigy
Penbutolol	Hoechst (Frankfurt, Germany)
Pindolol	Durascan (Odense, Denmark)
Pindolol	Benzon Pharma
Pindolol	NM Pharma (Sundbyberg, Sweden)
Pindolol	Dumex (Copenhagen, Denmark)
Propranolol	Sigma (St. Louis, MO, USA)
Sotalol	Bristol-Myers (Evansville, IN, USA)
Tertatolol	Servier (Orleans, France)
Timolol	Merck, Sharp & Dohme (Rahway, NJ, USA)



$$\text{R}_1 = \text{OAr}$$

$$= \text{Ar (Sotalol)}$$

$$\text{R}_2 = \text{CH}(\text{CH}_3)_2$$

$$= \text{C}(\text{CH}_3)_3 \text{ (Bunolol, Carteolol, Penbutolol, Tertatolol, Timolol)}$$

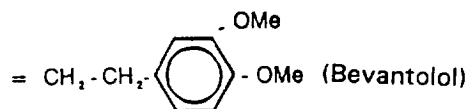


Fig. 1. Structures of the β -blockers investigated.

Table 2
Chromatographic results from the separation of 18 β -blocking substances on the Chiralcel OD stationary phase

Compound	λ (nm)	Eluent ^a	$k'(1)$	$k'(2)$	α	R_s	
Acebutolol	320	G	2.77	3.19	1.15	1.6	
Alprenolol	275	A	0.35	1.24	3.54	10.9	
Atenolol	275	C	0.94	1.75	1.86	4.4	
Betaxolol	275	A	0.52	1.57	3.02	10.8	
Bevantolol	275	E	0.97	4.10	4.23	9.8	
Bisoprolol	275	A	0.69	1.38	2.00	6.9	
Bunolol	267	A	0.71	1.42	2.00	6.6	
Carazolol	253	C	1.87	2.32	1.24	1.7	
Carteolol	275	C	0.48	0.95	1.98	4.1	
Metipranolol	275	B	1.08	1.31	1.21	2.2	
Metoprolol	275	A	0.69	2.02	2.93	11.8	
Oxprenolol	275	D	0.32	1.62	5.06	12.9	
Penbutolol	270	A		0.60			
Pindolol	265	F	0.25	1.66	6.64	7.6	
Propranolol	290	C	0.90	1.52	1.69	4.6	
Sotalol	254	Eluted as a single peak with all mobile phases tested					
Tertatolol	290	D	0.32	1.69	5.28	10.5	
Timolol	300	B	0.85	1.07	1.26	2.5	

^a Eluents: A = hexane–2-propanol–DEA (80:20:0.1); B = hexane–2-propanol–DEA (90:10:0.1); C = hexane–2-propanol–DEA (60:40:0.1); D = hexane–2-propanol–DEA (40:60:0.1); E = hexane–2-propanol–DEA (10:90:0.1); F = hexane–2-propanol–DEA (20:80:0.1); G = hexane–ethanol–DEA (90:10:0.1). DEA = diethylamine.

phenylcarbamate) column and the mobile phases listed. The mobile phases chosen for the Chiralcel OD stationary phase were various mixtures of hexane and 2-propanol with the addition of 0.1% diethylamine (see Table 2). These mobile phases were as suggested by the manufacturer of the column. Samples, as salts or free bases, were dissolved in the mobile phase. For the separation studies the sample concentration was 1 mg/ml, 20 μ l were injected and the flow-rate was 0.5 ml/min. For information on detection wavelengths, see Table 2. It appears from the results that this column is capable of separating fifteen of the eighteen substances under these conditions. Racemic acebutolol was not separated satisfactorily, racemic sotalol eluted as a single peak and penbutolol was only available in the *S*-form. A chromatogram of racemic timolol is shown in Fig. 2.

Some workers have investigated chiral recognition mechanisms with this stationary phase. In some cases altered capacity factors and reversed retention orders were achieved by the addition of organic alcohols other than 2-propanol to the mobile phase, in this way optimizing the separations [41,43]. Such an approach was attempted with the two compounds that were not separated satisfactorily, acebutolol and sotalol. The alcohols used as alternative modifiers were *tert*-butanol, *n*-butanol, *n*-propanol, ethanol and methanol. For racemic acebutolol an improved

separation could be obtained when using hexane–ethanol–diethylamine (90:10:0.1), resulting in $R = 1.6$ (see Table 2). For sotalol, the use of these modifiers in the mobile phase did not offer any improvements. The poor separation of sotalol using the Chiralcel OD column may be due to the lack of an oxygen atom in connection with the aromatic group close to the chiral centre, as compared with the other β -blocking substances.

The possible use of Chiralcel OD as a stationary phase for the determination of optical purity in the two β -blocking drug substances marketed as the pure enantiomeric forms, (*S*)-timolol and (*S*)-bunolol, was validated with respect to specificity, reproducibility, accuracy, linearity, limit of detection and ruggedness, in accordance with the current EEC guideline [47]. For both timolol and bunolol the *R*-form eluted before the *S*-form. For both compounds the chromatographic systems given in Table 2 were used.

Fig. 3 shows a chromatogram of (*S*)-timolol with the addition of 1% of (*R*)-timolol. Linearity of the detector response of (*R*)-timolol added to

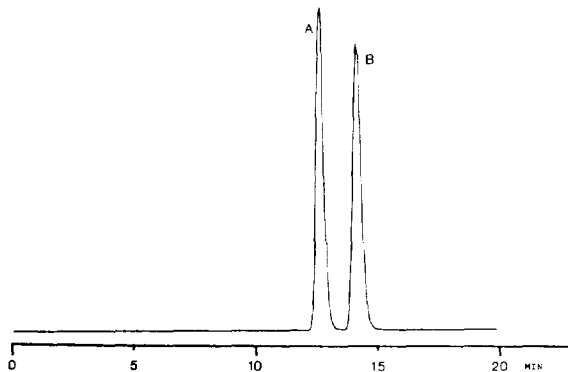


Fig. 2. Chromatogram of racemic timolol. Column, Chiralcel OD; injection volume, 20 μ l; sample concentration, 1 mg/ml; detection wavelength, 300 nm. Peaks: A = (*R*)-timolol; B = (*S*)-timolol.

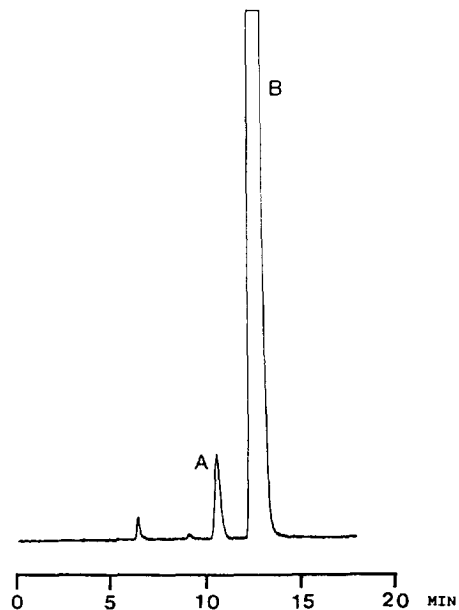


Fig. 3. Chromatogram of (*S*)-timolol spiked with 1% (*R*)-timolol. Column, Chiralcel OD; injection volume, 20 μ l; sample concentration, 1 mg/ml; detection wavelength, 300 nm. Peaks: A = (*R*)-timolol; B = (*S*)-timolol.

the (*S*)-form was observed up to a content of 8%. The regression equation (area counts versus percentage added) was $y = 1\,189\,450x + 8274$ ($r = 1.0000$). The corresponding result for (*S*)-bunolol was $y = 21\,254x + 2546$ ($r = 1.0000$). The reproducibility was investigated at a level of 1% of the *S*-form added to the *R*-form; calculations from six determinations resulted in relative standard deviations (R.S.D.s) of 0.84% for (*R*)-timolol and 1.9% for (*R*)-bunolol. The limits of detection were estimated to be ca. 0.1% and 0.05% for (*S*)-timolol and (*S*)-bunolol, respectively.

It was found that the sample solutions were stable for at least 1 week, i.e., no extra peaks appeared in the chromatograms and no detectable racemization occurred in solutions stored at room temperature. The performance of the column was unaltered after several hundred injections and exposure to various mobile phases as described above.

3.2. AGP α -glycoprotein

A thorough evaluation of the ability of this stationary phase to separate β -blocking substances has been reported [13]. It was stated that this column is able to separate a wide range of β -blocking substances. In the present investigation, a procedure suggested by the manufacturer of the column was followed, using mixtures of 2-propanol and 10 mM aqueous phosphate buffers as the mobile phase for the separation of β -blockers. A volume of 20 μ l of a sample concentration of ca. 0.02 mg/ml was injected, corresponding to an injected amount of 1–2 nmol of substance. The ability of the α -glycoprotein stationary phase to determine the *R*-form as an impurity in the *S*-form was evaluated for both timolol and bunolol. The separation of enantiomeric forms of timolol and bunolol was investigated and the use of a mixture of 1.5% 2-propanol in 20 mM phosphate buffer (pH 6.5), delivered at a flow-rate of 0.9 ml/min. These conditions were found to be the optimum when considering both the separation and a reasonable retention time. Detection wavelengths of 220 nm for bunolol and 300 nm for timolol were found to

provide the best results. The resolutions for the enantiomers of bunolol and timolol were 1.8 and 2.0, respectively, under these conditions.

Fig. 4 shows a chromatogram of timolol. For both timolol and bunolol the *R*-form eluted before the *S*-form. As described above for the Chiralcel OD column, validation studies were performed according to the EEC guideline [47]. Linearity of the detector response of (*R*)-timolol added to the *S*-form was observed up to a content of 10%. The regression equation (area counts versus percentage added) was $y = 8643x + 1283$ ($r = 0.9997$); the large intercept on the abscissa indicated a small content of the *R*-enantiomer in the (*S*)-timolol raw material. The corresponding results for (*S*)-bunolol were $y = 8296x + 38$ ($r = 0.9998$). The reproducibility was investigated at a level of 1% of the *S*-form added to the *R*-form; calculations from six determinations resulted in R.S.D.s of 4.9% for (*R*)-timolol and 7.6% for (*R*)-bunolol. The limits of detection were estimated to be ca. 0.25% for both (*S*)-timolol and (*S*)-bunolol.

A serious drawback to the use of the AGP stationary phase was discovered during longer series (ca. 6–10 h) of analysis, with the separation power gradually deteriorating, i.e., severe

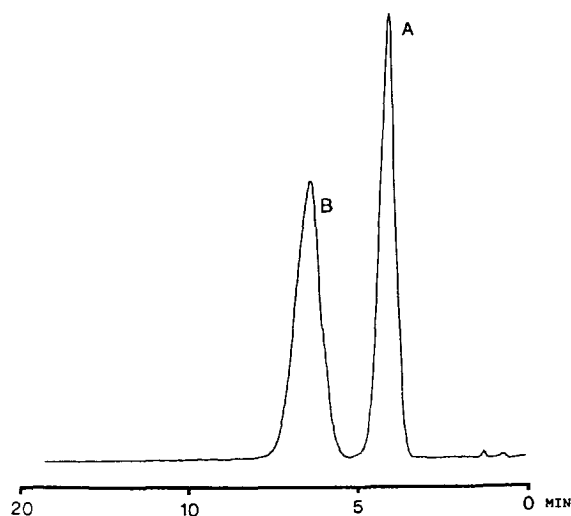


Fig. 4. Chromatogram of racemic timolol. Column, AGP; injection volume, 10 μ l, sample concentration, 20 mM; detection wavelength, 300 nm. Peaks: A = (*R*)-timolol; B = (*S*)-timolol.

peak broadening and peak splitting were observed. The column could be regenerated by flushing with a 25% solution of 2-propanol for 2 h followed by flushing with water for 1 h. Accordingly, overnight utilization of the column employing automatic injection devices was of limited use.

3.3. β -Cyclodextrin

This column has been used with medium-polarity mobile phases, consisting of mixtures of acetonitrile, methanol, acetic acid and triethylamine, the separation of some β -blocking substances being achieved [25]. Apart from that, no work has been published which indicated that this stationary phase could provide the separation of β -blockers for analytical purposes. Therefore, the separation of the investigated β -blockers was attempted using various compositions of the above-mentioned mobile phase. The sample concentration was 1 mg/ml, the volume injected was 20 μ l and flow-rate was 1 ml/min. It should be noted that the order of retention is reversed compared with the Chiralcel OD and the AGP stationary phases, i.e., with the β -cyclodextrin column the *S*-form elutes prior to the *R*-form. The detection wavelengths and the results obtained are given in Table 3. Fig. 5

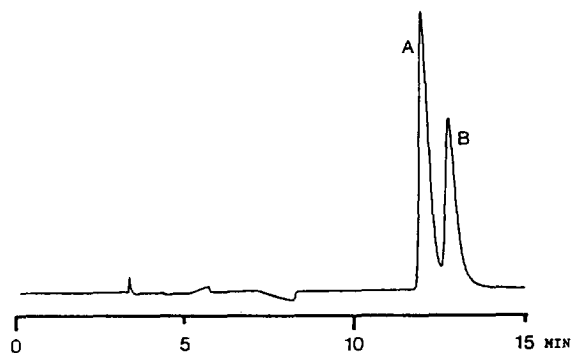


Fig. 5. Chromatogram of 1:1 mixture of (*R*)- and (*S*)-bunolol. Column, Cyclobond I; injection volume, 20 μ l; sample concentration, 0.5 mg/ml each; detection wavelength, 300 nm. Peaks: A = (*S*)-timolol; B = (*R*)-timolol.

shows a chromatogram for the separation of a 1:1 mixture of bunolol.

An attempt was made to separate all eighteen β -blockers, but for the eight substances not listed in Table 3 only very poor or no separations were obtained. The results in Table 3 show the chromatographic parameters for the ten β -blockers able to be separated with the β -cyclodextrin stationary phase. It is clear that the separation power is low with this phase, with α -values in the range 1.04–1.10. In order to evaluate the usefulness of this stationary phase in determining the *R*-form as an impurity in the *S*-form, the reproducibility for (*R*)-timolol added to the *S*-form at

Table 3
Chromatographic results for the compounds that could be successfully separated on the β -cyclodextrin (Cyclobond I) stationary phase

Compound	λ (nm)	Eluent ^a	k' (1)	k' (2)	α	R_s
Alprenolol	275	A	3.6	3.75	1.04	0.87
Betaxolol	275	B	3.9	4.2	1.07	1.14
Bisoprolol	275	A	5	5.23	1.05	0.70
Bunolol	267	B	2.93	3.19	1.09	1.52
Carazolol	253	B	5.13	5.47	1.07	1.16
Carteolol	275	A	7.27	7.67	1.05	0.96
Metoprolol	275	B	4.2	4.66	1.1	1.9
Propranolol	290	B	3.7	4.0	1.06	1.25
Tertatolol	290	B	3.1	3.3	1.06	1.14
Timolol	300	B	2.9	3.3	1.1	1.96

^a Eluents: A = acetonitrile–methanol–acetic acid–triethylamine (99:1:0.24:0.36); B = acetonitrile–methanol–acetic acid–triethylamine (97:3:0.24:0.36).

a level of 1% was determined; the R.S.D. from six determinations was 12.5%. For bunolol, the limit of detection of the *R*-form in the *S*-form was ca. 2.5%, an unacceptably high limit of detection which to some extent is due to the fact that the impurity peak, i.e., the *R*-form, elutes after the principal peak, without being sufficiently separated. The high values for the reproducibility and the detection limit show that the β -cyclodextrin phase is not very useful compared with the two other phases tested.

4. Conclusions

Analysis of commercial samples of (*S*)-bunolol and (*S*)-timolol has shown that these contain 0.2–0.8% of the *R*-isomer. Thus, for a useful method for the determination of one enantiomer as an impurity in the other, the detection limit should be not more than 0.1% of the *R*-isomer, in order to provide adequate routine analyses of β -blocker raw materials. These investigations have shown that Chiralcel OD is the best suited for the determination of the enantiomeric purity of β -blockers. Sotalol was the only β -blocker that this column was unable to separate. Otherwise, the column provided separations with sufficiently good peak shapes and with reasonable times of analysis for practical analytical purposes. The column was found to be very stable when used for extended periods of analysis. Further, this stationary phase has been successfully applied to the determination of enantiomeric purity in a finished dosage form [48].

The AGP column also provides acceptable separations of β -blocking substances and with acceptably low detection limits for the determination of enantiomeric purity, even though the amount of substance injected is very small. The AGP column, however, seems to be less rugged in normal laboratory use, with peak broadening occurring after only 6–10 h of use, this being a serious drawback for routine analytical purposes. The β -cyclodextrin column does not provide sufficiently good separations to be used for the determination of the optical purity of β -blockers. Another disadvantage of the β -cyclodextrin

phase, when used to separate timolol and bunolol, is that the *S*-form elutes before the *R*-form, the impurity peak eluting on the tail of the main peak, causing an unacceptably high limit of detection.

Acknowledgements

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