Journal of Chromatography, 636 (1993) 231–241
Elsevier Science Publishers B.V., Amsterdam

CHROM 24 857

Chiral separations of β-blocking drug substances using derivatization with chiral reagents and normal-phase high-performance liquid chromatography

Lars Olsen*, Kirsten Brønnum-Hansen, Per Helboe, Gorm Herlev Jørgensen and Sonja Kryger

Chemical Laboratory, National Board of Health, 378 Frederikssundsvej, DK-2700 Brønshøj (Denmark)

(First received June 19th, 1992; revised manuscript received December 8th, 1992)

ABSTRACT

Eighteen β-blockers currently on sale in Denmark were investigated for the possible separation of the enantiomers by use of normal-phase HPLC following derivatization with three different chiral derivatization agents: (−)-camphanic acid chloride (*S*)-(−)-1-phenylethyl isocyanate and 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate. Chromatography was performed using a 5-μm silica column (120 × 4.6 mm I.D.) with mixtures of dichloromethane, *n*-heptane and methanol as eluents. The results showed that all the β-blockers available in both enantiomeric forms (seventeen) can be baseline separated by at least one of the three procedures. The purpose of the investigations was an evaluation of the various approaches concerning the possibilities of method standardization.

INTRODUCTION

In recent years, the possibilities of resolving enantiomers by high-performance liquid chromatography (HPLC) have greatly improved. Compared with the traditional determination of optical rotation, these techniques offer an ability for the direct and accurate measurement of enantiomeric purity.

There are three possible approaches to enantiomeric resolution by use of HPLC: (1) separation on a chiral stationary phase and using an achiral eluent, (2) derivatization with a chiral reagent and separation of the resulting diastereomers using an achiral stationary phase and eluent and (3) separation on an achiral stationary phase by use of a chiral eluent.

A prerequisite for using HPLC methods for the evaluation of the optical purity, *e.g.*, in national and international pharmacopoeias, is the possibility of standardizing the analytical methods. Therefore, investigations to evaluate the above three possible approaches in that context were considered necessary.

The therapeutic group of β-adrenergic blocking agents was chosen as a model class. This group includes a series of chemically closely related substances, as can be seen from Fig 1. Further, the β-blocking agents exhibit enantiospecific therapeutic activity, usually with the *S*-form possessing the β-blocking activity (an exception being SOT), even though most β-blockers until now have been used as the racemic mixture. This specificity could suggest an increasing demand for standardized methods for determining the enantiomeric purity of these compounds.

The three different approaches mentioned above have been used previously on β-blocking agents. (1) The most feasible choice among the

* Corresponding author.

0021-9673/93/$06.00

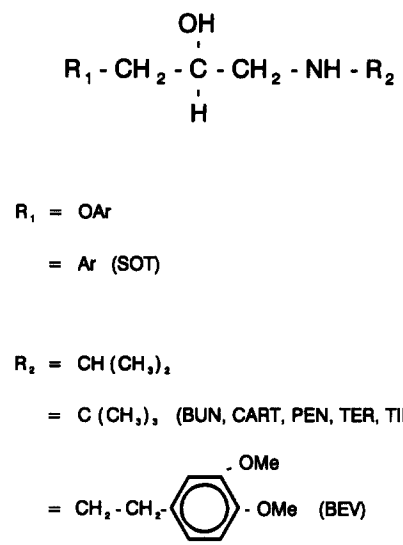

Fig. 1. Molecular structures of the eighteen β-blocking agents investigated.

chiral stationary phases appears to be cellulose tris(3,5-dimethylphenylcarbamate) [1–8], but the use of other stationary phases has been reported, such as the Pirkle type [9–11], immobilised α_1-acid glycoprotein [12–14] and another type of immobilized acid glycoprotein, ovomucoid [15]. The use of β-cyclodextrin [16] and immobilized bovine serum albumin (BSA) [17,18] has also been investigated but the results obtained seem less promising owing to low separation factors or considerable peak broadening. (2) Several examples of separating β-blockers following chiral derivatization have been described. In most instances the separation of the resulting diastereomers has been performed using reversed-phase HPLC: (*S*)-(−)- or (*R*)-(+)-1-phenylethyl isocyanate [(*S*)- or (*R*)-PEI] [19–26], (*R*)-(−)-1-(1-naphthyl)ethyl isocyanate (R-NEI) [27,28], 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (D-GITC) [29–32] and 2,3,4-tri-O-acetyl-α-D-arabinopyranosyl isothiocyanate [29], N-trifluoroacetyl-1-prolyl chloride [33], (*R*,*R*)-O,O′-diacetyltartaric acid anhydride [34,35], *tert.*-butoxycarbonyl-L-leucine anhydride [36,37], and different chloroformates [38–42]. A limited number of publications describing the use of normal-phase HPLC for the separation of the diastereomeric derivatives can be found with (*S*)-PEI [43] and (*S*)-NEI [44,45]. (3) The third approach, the use of chiral eluents, has also been attempted on β-blocking agents, *e.g.*, by adding to the eluent (+)-10-camphorsulphonate [46] and N-benzoxycarbonyl-glycyl-L-proline [47,48].

Further, a comprehensive review on the chromatography in general of β-blocking agents, including a section on chiral separation also dealing with the various approaches, has recently been published [49].

No publications so far have addressed the problem of standardization of methods and only a few have discussed the suitability of determining optical purity. The main object of most investigations has been to demonstrate the separation power of the approach investigated. In this work, eighteen β-blockers currently on sale in Denmark were investigated for the possible enantiomeric separation and purity testing using an easily standardizable method. For this purpose we used normal-phase HPLC following derivatization with three different chiral derivatization reagents, (−)-camphanic acid chloride [(−)-CACl], (*S*)-PEI and D-GITC. The use of (−)-CACl for the chiral separation of β-blockers has not previously been reported. With the proper set of reaction conditions, both isocyanates and thioisocyanates are known to react selectively with the amines of β-blockers owing to the relatively slow reaction of the hydroxy group, whereas the acid chloride was likely to show comparable reactivity towards the hydroxy group of the β-blockers, so that difficulties regarding selectivity of the reactions could be expected. However, this reagent is comparatively inexpensive and was included in the investigations mainly for this reason.

The number of investigations published so far using normal-phase chromatography following diastereomeric derivatization are limited compared with the number of papers using reversed-phase chromatography.

EXPERIMENTAL

Chemicals

In Table I are listed the compounds investigated and the companies from which they were obtained. Other drug substances, including

TABLE I

INVESTIGATED β-BLOCKING AGENTS AND THEIR SOURCES

Compound	Abbreviation	*R*-	*S*-	Rac.	Company
Acebutolol	ACE	+	+	+	Rhône-Poulenc (Dagenham, UK)
Alprenolol	ALP	+	+	+	Hässle (Mölndal, Sweden)
Atenolol	ATE			+	ICI (Runcorn, UK)
Atenolol				+	Benzon Pharma (Hvidovre, Denmark)
Betaxolol	BET	+	+	+	MEDA (Herlev, Denmark)
Betaxolol		+	+	+	Searle (Mölndal, Sweden)
Bevantolol	BEV	+	+	+	Parke-Davis (Ann Arbor, MI, USA)
Bevantolol		+	+	+	Benzon Pharma (Hvidovre, Denmark)
Bisoprolol	BIS			+	Merck (Darmstadt, Germany)
Bunolol	BUN	+	+		Allergan (Irvine, CA, USA)
Carazolol	CARA			+	Upjohn (Crawley, UK)
Carteolol	CART	+	+	+	Ercopharm (Vedbæk, Denmark)
Metipranolol	METI	+	+	+	Ciba-Geigy (Basle, Switzerland)
Metoprolol	METO		+	+	Hässle
Oxprenolol	OXP	+	+	+	Ciba-Geigy
Penbutolol	PEN		+		Hoechst (Frankfurt, Germany)
Pindolol	PIN			+	Durascan (Odense, Denmark)
Pindolol				+	Benzon Pharma (Hvidovre, Denmark)
Pindolol				+	NM Pharma (Sundbyberg, Sweden)
Pindolol				+	Dumex (Copenhagen, Denmark)
Propranolol	PRO	+	+		Sigma (St. Louis, MO, USA)
Sotalol	SOT	+	+	+	Bristol-Myers (Evansville, IN, USA)
Tertatolol	TER			+	Servier (Orleans, France)
Timolol	TIM	+	+	+	Merck, Sharp & Dohme (Rahway, NJ, USA)

racemic propranolol (*rac*-PRO), were of pharmacopoeial quality. (*R*)-PEI, (*S*)-PEI and (−)-CACl were of ChiraPur quality and obtained from Fluka (Buchs, Switzerland) and D-GITC from Polysciences (Warrington, PA, USA). All other chemicals were of analytical-reagent grade from Merck (Darmstadt, Germany).

Apparatus

The chromatographic systems consisted of a Kontron Model 410 or Model T-414 LC pump, a Rheodyne Model 7125 injection valve and a Kontron Model 735 LC, Cecil Model CE 2012 or CE 212 or LDC Spectromonitor II 1202 UV detector. Chromatograms were recorded on a Kipp & Zonen Model BD-8 recorder. Retention data were collected on a Hewlett-Packard Model 3359A laboratory data system.

Chromatography

All normal-phase experiments were performed on 120 × 4.6 mm I.D. columns from Knauer (Berlin, Germany) packed by the dilute slurry technique with 5-μm LiChrosorb Si 60, silica (Merck).

The eluents for normal-phase chromatography were mixtures of methanol, dichloromethane and heptane (see Table III). Prior to use the columns were activated by elution with a series of solvents, *viz.*, water, methanol, acetone, ethyl acetate, dichloromethane and heptane.

Reversed-phase chromatography was performed on 120 × 4.6 mm I.D column from Knauer packed by the dilute slurry technique with 5-μm LiChrosorb RP-18. The eluent was 2 m*M* sodium 1-dodecylsulphonate in triethylamine–glacial acetic acid–methanol–water (0.2:0.2:75:25)

Derivatization procedure

(R)- and (S)-PEI. The compounds to be derivatized, either as a salt or free base, were dissolved in dichloromethane–triethylamine (10:1), typically at a concentration of 5 mg/ml.

To 200 μl (or a volume containing a sample amount of about 1 mg) were added 50 μl of a solution containing 3 mg of the derivatization agent in dichloromethane. The mixture was allowed to react for 5 min at room temperature and evaporated to dryness using a stream of nitrogen. The residue was dissolved in 800 μl of dichloromethane–heptane (1:1).

D-GITC. The procedure was as described for PEI, with the exception of a reaction time of 15 min. The residue was dissolved in 1 ml of heptane–methanol (40:1).

(−)-CACl. The procedure was as described for PEI, with the exception of the addition of 10 mg of derivatization agent (in 50 μl of solvent) and reaction times of 30–60 min. The residue was dissolved in 1.5 ml of dichloromethane–heptane (2:1).

RESULTS AND DISCUSSION

Chromatography

Preliminary investigations showed that the selectivity towards the diastereomeric derivatives was strongly influenced by the relative composition of the eluent. This effect when using mixtures of methanol, dichloromethane and heptane is illustrated in Table II with the results obtained during initial experiments with (*S*)-PEI derivatives of *rac*-PRO. It appears that the chromatographic behaviour of the diastereomeric derivatives varies from complete co-elution to baseline separation. Other alcoholic modifiers were investigated such as 2-propanol and higher alcohols, but the combination of methanol, dichloromethane and heptane was found to be the most suitable for the separation of all three types of derivatives investigated with respect to selectivity and peak shape.

Fig. 2 shows chromatograms of *rac*-PRO derivatized with the three different reagents. A detailed presentation, giving capacity factors (k'), separation factors (α), resolution (R_s) and asymmetry factors (A_s) of the chromatographic results obtained by the above-mentioned procedures is shown in Table III. It appears that with the exception of PEN, which was available as the *S*-form only, all the racemic β-blockers could be baseline separated as (*S*)-PEI and D-GITC derivatives. Using (*S*)-PEI, resolutions of not less than 2.9 were obtained (except for ATE and BEV, with R_s values of 1.6 and 1.8, respectively) with k' below 6 and acceptable values of A_s. Correspondingly, when using D-GITC, resolutions of not less than *ca.* 1.5 were found in all instances with asymmetry at an acceptably low level. Even though the retentions, with k' values up to *ca.* 15, were higher than for the (*S*)-PEI derivatives, the D-GITC derivatives were in most instances separated within an acceptable time of analysis. For both reagents, the order of elution of the derivatized β-blocking agents was identified. For both (*S*)-PEI and D-GITC, the *R*-forms of the β-blocking agents eluted first [or the *S*-forms, when using (*R*)-PEI]. The only exception to this behaviour was found with SOT,

TABLE II

RETENTION (CAPACITY FACTORS, k'), SEPARATION FACTORS, α, RESOLUTION, R_s, AND ASYMMETRY, A_s, OF (*S*)-PEI-DERIVATIZED *rac*-PRO USING NORMAL-PHASE HPLC WITH SIX DIFFERENT ELUENTS

Derivatization procedure as described under Experimental. Detection wavelength, 290 nm.

Eluent composition			$k'(1)$	$k'(2)$	α	R_s	A_s
Methanol	Dichloromethane	Heptane					
1.0	85.0	14.0	2.55	3.54	1.39	4.9	3.0
1.2	70.0	18.8	2.00	2.85	1.43	5.4	1.9
1.5	50.0	48.5	1.77	2.50	1.41	5.3	1.5
1.5	30.0	68.5	2.77	3.61	1.30	4.9	1.6
2.0	20.0	78.0	2.93	3.45	1.18	3.1	1.2
4.0	0	96.0	8.00	8.00	1.00	0.0	1.5

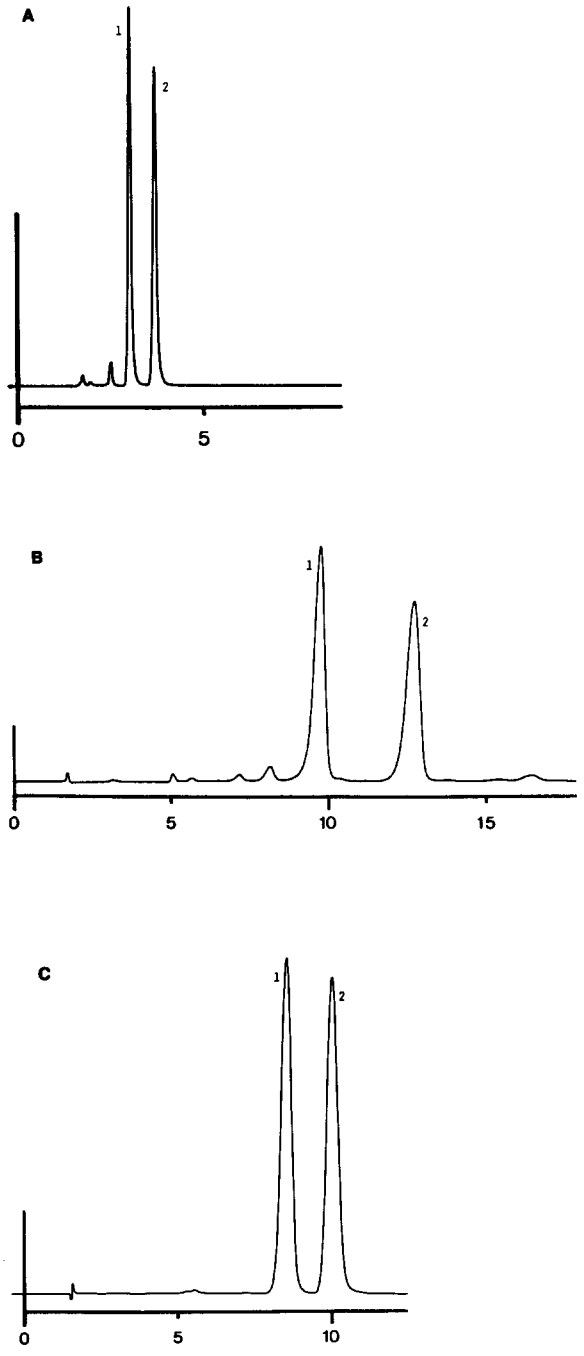

Fig. 2. Chromatograms of *rac*-PRO derivatized with (A) (*S*)-PEI, (B) D-GITC and (C) (−)-CACl as described under Experimental. Column: 5-μm LiChrosorb Si 60 (120 × 4.6 mm I.D.). Eluent: methanol–dichloromethane–heptane, (A) (1.5:50:48.5), (B) (3:10:90) and (C) (0.35:60:40). Detection at 290 nm. Peaks: 1 = (*R*)-PRO; 2 = (*S*)-PR. Time scales in Figs. 2–5 in min.

which for both reagents showed an opposite order of elution. This is caused by the sequence rules for naming the configurations as *R* or *S* because, as can be seen from Fig. 1, the aryloxymethyl ligand otherwise present in the molecular structures of β-blockers is replaced with aryl in this compound.

In Table III are also listed results obtained using (−)-CACl as the chiral derivatization agent. It can be seen that the polarity of the eluent used for nine of the β-blocking agents was much lower than for the eluents used in connection with the other reagents and the remaining compounds derivatized with (−)-CACl. A possible explanation to the apparent difference in the lipophility of the derivatives is further discussed below.

Derivatization

(S)-PEI and D-*GITC.* The molecules of the β-blockers investigated exhibit two groups available for derivatization, a secondary hydroxy and a secondary amino group.

It has been shown previously for PRO that only the amino group is derivatized, resulting in urea derivatives, when (*R*)-PEI is used as reagent [19]. Correspondingly, the isothiocyanate group of D-GITC is known to react selectively with the amino group [29,50–52]. As the derivatization procedures used in this investigation are slightly modified compared with previously published methods, the reactivity towards alcohols and amino groups was examined.

During an earlier study of (*S*)-PEI as a potential chiral derivatization agent for separation of norgestrel enantiomers, the reactivity towards the secondary alcohol was found to be considerably higher in dichloromethane than in other more polar solvents such as acetonitrile [53], although a high temperature and long reaction time were required. Therefore, in order to obtain a full turnover of the compounds in the synthesis, it was assumed that dichloromethane should be preferred as the solvent for both reagents. This was supported by derivatization experiments on *rac*-PRO. By using the reversed-phase HPLC system described under Experimental, it was possible to determine the amount of underivatized *rac*-PRO, with a detection limit of

TABLE III

RETENTION (CAPACITY FACTOR, k'), SELECTIVITY, α, RESOLUTION, R_s, AND ASYMMETRY, A_s, OF EIGHTEEN β-BLOCKING AGENTS DERIVATIZED WITH THREE DIFFERENT CHIRAL REAGENTS

Derivatization as described under Experimental. Eluents: methanol–dichloromethane–heptane, (A) (1.5:50:48.5); (B) (2.5:49.5:48); (C) (3.5:49:47.5); (D) (3:10:90); (E) (3:20:80); (F) (3:25:75); (G) (3:30:70); (H) (3:50:50); (I) (0.35:60:40); (J) (1.5:60:40); (K) (2:60:40).

Compound	λ (nm)	(S)-PEI						D-GITC						λ (nm)	(−)-CSCl					
		Eluent	$k'(1)$	$k'(2)$	α	R_s	A_s	Eluent	$k'(1)$	$k'(2)$	α	R_s	A_s		Eluent	$k'(1)$	$k'(2)$	α	R_s	A_s
Acebutolol	320	B	4.10	4.81	1.17	4.0	1.5	G	6.99	7.80	1.12	1.4	1.0							
Alprenolol	275	A	1.10	1.57	1.43	4.8	1.0	D	4.96	6.85	1.38	4.7	0.7	275	I	5.94	8.29	1.40	4.8	1.4
Atenolol	275	C	6.44	7.05	1.09	1.6	1.5	H	15.7	17.2	1.10	1.7	1.4							
Betaxolol	275	A	1.70	2.34	1.37	4.4	1.6	D	9.15	12.09	1.32	4.4	0.9	275	I	10.01	11.82	1.18	2.0	1.3
Bevantolol	275	A	1.55	1.79	1.15	1.8	1.0	F	2.10	2.49	1.19	1.9	1.6	275	I	7.19	8.75	1.22	2.5	1.2
Bisoprolol	275	A	1.90	2.51	1.32	3.8	1.6	D	9.40	11.88	1.26	3.6	0.7							
Bunolol	267	A	2.80	4.23	1.51	6.0	0.9	E	4.70	5.45	1.16	1.4	0.9	267	K	1.23	1.63	1.33	2.4	1.7
Carazolol	285	A	1.83	2.40	1.31	3.8	1.6	F	5.21	6.34	1.22	2.9	1.2	253	I	6.35	7.39	1.16	2.2	1.2
Carteolol	275	B	2.63	3.23	1.23	3.2	1.4	F	12.20	13.60	1.11	1.4	1.0	275	K	5.25	6.38	1.21	1.9	1.5
Metipranolol	275	A	1.63	2.36	1.44	5.1	1.4	D	7.09	9.40	1.33	4.5	0.7	275	I	7.65	8.90	1.16	1.9	1.9
Metoprolol	275	A	2.06	2.78	1.35	4.2	1.2	D	8.85	11.64	1.32	4.1	0.9	275	I	13.65	15.80	1.15	1.9	1.3
Oxprenolol	275	A	1.45	1.81	1.25	2.9	1.0	D	6.47	7.86	1.21	2.7	0.8	275	I	5.92	7.40	1.25	2.8	1.3
Penbutolol	270	A	1.06				1.1	D	3.18				1.2	270	K	0.78				0.9
Pindolol	265	A	2.45	3.39	1.38	5.1	1.1	F	6.76	7.58	1.12	1.8	0.9	265	I	9.75	11.88	1.22	2.9	1.1
Propranolol	290	A	1.08	1.56	1.44	4.3	1.3	D	5.86	7.99	1.36	4.2	0.8	290	I	4.60	5.60	1.23	2.5	1.1
Sotalol	254	B	3.31	3.97	1.20	3.4	1.7	F	15.8	17.7	1.12	1.4	0.8							
Tertatolol	290	A	0.78	1.06	1.36	3.3	1.2	D	5.65	6.52	1.15	2.0	1.1	290	I	1.94	2.53	1.30	1.7	1.8
Timolol	300	A	1.49	2.27	1.52	5.5	1.3	E	2.79	3.59	1.29	2.4	1.2	300	J	2.43	3.39	1.39	3.3	1.4

0.1% of the amount employed in the reaction. By using the suggested procedures for (*S*)-PEI and D-GITC, no trace of underivatized *rac*-PRO could be detected, indicating complete turnover in the synthesis. When using acetonitrile as the solvent up to 15–20% of underivatized *rac*-PRO was observed when (*S*)-PEI was used as the reagent.

There was no indication that the reactions of the two reagents were not selective towards the amino group. However, for (*S*)-PEI, on increasing the reaction time to several hours it was possible to detect an extra set of peaks as the result of a subsequent carbamate reaction of the hydroxy group in significant amounts. Under the conditions described here less than 0.1% of these double derivatives, eluted early in the chromatograms, was seen.

To investigate the selectivity of (*S*)-PEI further, *rac*-PRO was derivatized with this reagent and subsequently treated with (−)-CACl. The resulting chromatogram was compared with that obtained after (*S*)-PEI treatment alone. As can be seen from Fig. 3, the peaks from PRO–urea derivatives are completely removed after (−)-CACl treatment, which may indicate that the hydroxy group was fully accessible to the acid chloride reagent.

(−)-CACl. A preliminary investigation with *rac*-PRO as the model substance for (−)-CACl derivatization showed that relative to (*R*)- and (*S*)-PEI and D-GITC, a larger excess of reagent and longer reaction time were necessary in order to obtain a complete reaction with a single set of peaks in the chromatograms. This procedure, however, resulted in highly lipophilic derivatives compared with the ureas and thioureas of the other reagents. This was probably caused by the formation bis-derivatives as a result of acylation of both the amino and hydroxy group of PRO. As shown in Table III, nine of the eighteen β-blockers investigated could be separated by using eluent F. For these compounds, the *R*-form is eluted first.

For the remainder of the substances successfully separated, a stronger eluent had to be employed to elute the derivatives. A possible explanation of this could be that only mono derivatives were formed for these substances,

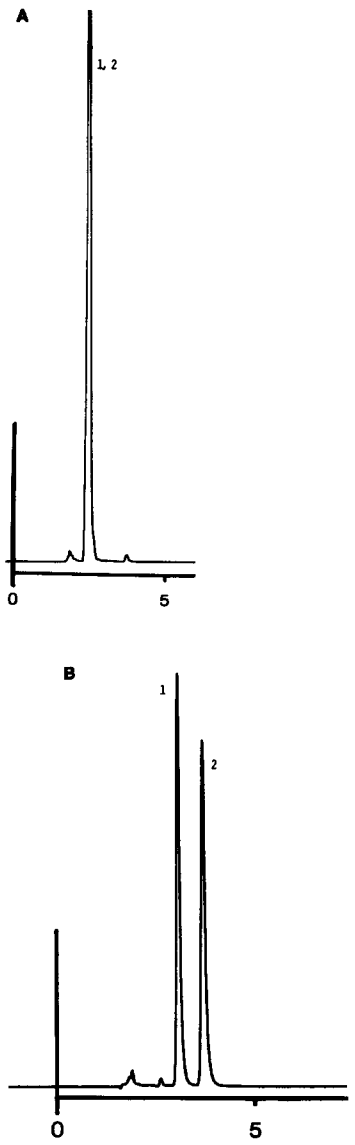

Fig. 3. Chromatograms of *rac*-PRO derivatized with (*S*)-PEI and subsequently with (A) (−)-CACl or (B) (*S*)-PEI alone. Derivatization procedures as described under Experimental. Column, 5-μm LiChrosorb Si 60 (120 × 4.6 mm I.D.); eluent, methanol–dichloromethane–heptane (1.5:50:48.5); detection at 290 nm. Peaks: 1 = (*R*)-PRO; 2 = (*S*)-PRO.

resulting in derivatives of higher polarity. For TIM, PEN, LEV and TER it appears from Fig. 1 that they all have *tert.*-butyl radicals attached to the amino function instead of the typical isopropyl group. The difference in the bulkiness of the moieties offers the possibility that only the

amine is accessible to the acid chloride, with a subsequent reaction of the hydroxy group sterically hindered. To investigate this assumption, *rac*-TIM (bearing a *tert.*-butyl radical) was treated with (*S*)-PEI and subsequently with (−)-CACl by the same procedure as described above for *rac*-PRO. As can be seen from Fig. 4, the addition of (−)-CACl resulted only in a partial disappearance of the *rac*-TIM urea peaks. Compared with the complete turnover of the *rac*-PRO ureas (*cf.*, Fig. 3), this indicates steric hindrance due to the bulkiness of the *tert.*-butyl radical compared with an isopropyl group. The *S*-form of the four compounds containing *tert.*-butyl eluted first, whereas the *R*-form of the isopropyl-containing compounds was eluted first.

From another group of β-blockers which all included an amide (ACE, ATE, CAR) or a sulphonamide moiety (SOT), it was only possible to obtain a successful separation of CAR, for which eluent G had to be employed. In this instance the *S*-form was eluted first.

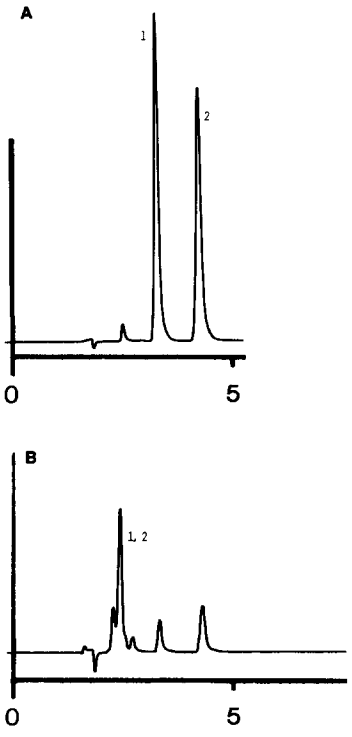

Fig. 4. Chromatograms of racemic timolol (TIM) derivatized with (*S*)-PEI and subsequently with (A) (−)-CACl or (B) (*S*)-PEI alone. Derivatization procedures as described under Experimental. Column, 5-μm LiChrosorb Si 60 (120 × 4.6 mm I.D.); eluent, methanol–dichloromethane–heptane (1.5:50:48.5); detection at 300 nm. Peaks: 1 = (*R*)-TIM; 2 = (*S*)-TIM.

The remaining β-blockers, including BIS, an isopropyl-containing compound without an amide group, could not be successfully separated using (−)-CACl as a reagent. No significant peaks were detected on testing a series of eluents with gradually increasing eluting strength with eluent G as the strongest. As the other two reagents investigated offered significant separations, no further investigations were performed.

Choice of chiral reagent

With a view to performing a further study of the applicability of normal-phase chromatography following chiral derivatization, a validation study was devised. The experience gained from the work so far performed with the different reagents enabled us to suggest the use of (*S*)-PEI as the most feasible choice. As criteria for this decision were considered parameters such as stability of the chromatography, including selectivity and asymmetry, but also stability of the derivatives and selectivity in the reaction of the reagent. During the following work the EEC guideline on analytical validation [54] was used.

Optical purity during synthesis

In order to use the approach of chiral derivatization for the determination of the optical purity of a substance, the enantiomeric purity of the reagent itself has to be of high optical purity, compared with the level of the substance being investigated. The optical purities of the reagents used in this investigation were guaranteed to be ≥99.5% for both (*S*)-PEI and (−)-CACl, according to the suppliers, but no information was available from the supplier of D-GITC. It is nevertheless likely that D-GITC was also of high purity, given the natural origin of the substance.

To examine the chiral purity of (*S*)- and (*R*)-PEI further, (*R*)- and (*S*)-PRO of very high purity were derivatized. By the method of standard additions only 0.1–0.2% of enantiomeric impurity was found for both enantiomers using the above-mentioned reagents. This indicates an optical purity of the reagents of at least 99.8%. The stability of the solutions used in the derivati-

zation procedure, (*S*)-PEI 60 mg/ml in dichloromethane, (*S*)-PRO 5 mg/ml in dichloromethane–triethylamine (10:1), and the solutions of the (*S*)-PRO derivatives of (*S*)-PEI were investigated. Using freshly prepared solutions of reagent and substrate, only about 0.1% corresponding to the peak of the (*R*)-PRO derivative was found with (*S*)-PRO derivatized with (*S*)-PEI. When stored for 14 days in a refrigerator the level was found to increase to about 0.5%. (*S*)-PRO solutions stored for 7, 45 and 105 days and derivatized with freshly prepared (*S*)-PEI showed enantiomeric impurity levels of 0.1%, 0.15% and 0.5%, respectively. Finally, a freshly prepared solution of (*S*)-PRO was derivatized with a solution of (*S*)-PEI that had been stored for 14 days in a refrigerator, and in this instance the enantiomeric impurity level reached about 1.0%. Overall the results indicate that the solution containing the β-blocker could be stored in refrigerator for up to 14 days, whereas the (*S*)-PEI solution and the solution of the derivative should be freshly prepared and analysed within hours.

Stability of the chromatographic systems used for the (S)-PEI derivatives

To investigate the stability of the chromatographic systems used for the (*S*)-PEI derivatives, the changes in the retention times of (*S*)-PEI propranolol derivatives were examined during a 3-week period. Each day a freshly prepared eluent was prepared. In 8 h, during a working day, the capacity factors usually increased by *ca.* 3%. This may be explained by a small evaporation primarily of dichloromethane, causing a slight decrease in the polarity of the eluent. Also an increase in temperature during the day was seen, which in normal-phase chromatography is expected to reduce retention.

On a day-to-day basis, using a freshly prepared eluent each day, the capacity factors were found to decrease by about 12% in 3 weeks. Throughout the period, a large number of samples were injected on to the column and, with the lipophilic nature of the system in mind, it could be expected that triethylammonium salts from the derivatization procedure would be adsorbed on the silica. This would result in a gradual deactivation of the solid phase, leading to lower retentions. By reactivating the column (see Experimental), the characteristics of the silica were found to be fully restored.

Validation of the methods

The possible use of the different reagents to monitor the optical purity in two β-blockers currently marketed as pure enantiomeric forms, (*S*)-TIM and (*S*)-BUN, was validated according to the EEC guideline [54] regarding specificity, reproducibility, accuracy, linearity and limit of quantification. For both compounds the chromatographic system given in Table III was used, with the exception that 310 nm was used as detection wavelength to reduce interference from reagent peaks. Fig. 5 depicts the results for (*S*)-BUN with the addition of 1% of the *R*-form. Linearity of the detector response for the *R*-form was ensured up to a content of 8%. The regression equations (area counts *versus* percentage) were $y = 5627x + 882$ ($r = 0.9996$) and $y =$

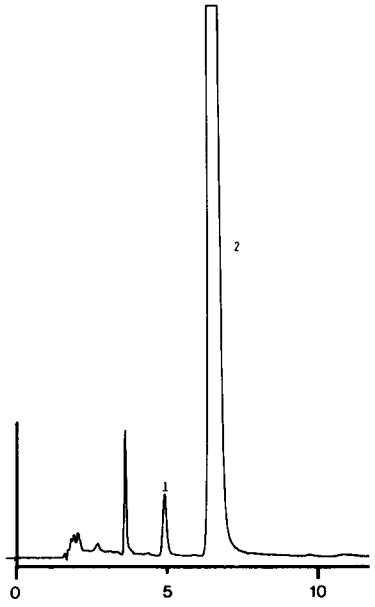

Fig. 5. Chromatogram of (*S*)-BUN (levobunolol), with 1% of (*R*)-BUN added, derivatized with (*S*)-PEI as described under Experimental. Column, 5-μm LiChrosorb Si 60 (120 × 4.6 mm I.D.); eluent, methanol–dichloromethane–heptane (1.5:50:48.5); detection at 310 nm. Peaks: 1 = (*R*)-BUN; 2 = (*S*)-BUN.

$9673x + 1466$ ($r = 0.9996$) for TIM and BUN, respectively, and the limits of detection were *ca.* 0.1 and 0.05 μg, respectively (corresponding to 0.1% and 0.05% of the amount in the main peak). By use of standard additions, the content of the *R*-form in the samples investigated was determined to *ca.* 0.15% for both (*S*)-TIM and (*S*)-BUN corresponding to the level resulting from the reagent. The reproducibility of the methods was examined for both TIM and BUN by use of a series of ten samples of the *S*-form to which were added 1% of the *R*-form and injecting each sample four times; $\bar{x}_{10} = 6333$, R.S.D. = 6.6% and $\bar{x}_{10} = 10\,605$, R.S.D. = 5.0% were found for TIM and BUN, respectively.

CONCLUSIONS

A number of HPLC methods based on normal-phase chromatography following chiral derivatization were investigated with a view to separating the enantiomers of eighteen different β-blocking agents currently on sale in Denmark. All β-blockers for which both enantiomeric forms were available were separated by at least one of the procedures described. One of the reagents, (*S*)-PEI, was selected as the most suitable choice for the separation of two β-blocking agents marketed as the enantiomeric forms (in both instances the *S*-form) with the intention of monitoring the optical purity. In both instances it was established that the procedures apply to the requirements described in the EEC guideline [54]. The limits of detection were determined to be *ca.* 0.1% and 0.05% for the *R*-form in (*S*)-TIM and (*S*)-BUN, respectively, with actual amounts found to be *ca.* 0.15% in the available samples for both compounds. In addition, the chromatographic system was found to exhibit satisfactory stability, this parameter being investigated with *rac*-PRO as a model substance. It is therefore concluded that the procedure of normal-phase HPLC following derivatization with (*S*)-PEI is well suited as a standardized method for the determination of the enantiomeric purity of β-blocking agents, *e.g.*, TIM and BUN.

REFERENCES

1 Y. Okamoto, M. Kawashima, R. Aburatani, K. Hatada, T. Nishiyama and M. Masuda, *Chem. Lett.*, (1986) 1237.
2 H. Takahashi, S. Kanno, H. Ogata, K. Kashiwada, M. Ohira and K. Someya, *J. Pharm. Sci.*, 77 (1988) 993.
3 A.M. Krstulovic and M.H. Fouchet, *J. Chromatogr.*, 452 (1988) 477.
4 C. Hartmann, D. Krauss, H. Spahn and E. Mutschler, *J. Chromatogr.*, 496 (1989) 387.
5 D.R. Rutledge and C. Garrik, *J. Chromatogr.*, 497 (1989) 181.
6 M.S. Ching, M.S. Lennard, A. Gregory and G.T. Tucker, *J. Chromatogr.*, 497 (1989) 313.
7 H.Y. Aboul-Enein and M.R. Islam, *Chirality*, 1 (1989) 301.
8 H.Y. Aboul-Enein and M.R. Islam, *J. Chromatogr.*, 511 (1990) 109.
9 I.W. Wainer and T.D. Doyle, *J. Chromatogr.*, 306 (1984) 405.
10 A.M. Dyas, M.L. Robinson and A.F. Fell, *Chromatographia*, 30 (1990) 73.
11 M. Ohwa, M. Akiyoshi and S. Mitamura, *J. Chromatogr.*, 521 (1990) 122.
12 J. Hermansson, *J. Chromatogr.*, 325 (1985) 379.
13 K. Balmer and B. Persson, *J. Chromatogr.*, 477 (1989) 107.
14 M. Enquist and J. Hermansson, *J. Chromatogr.*, 519 (1990) 285.
15 J. Haginaka, J. Wakai, K. Takahashi, H. Yasuda and T. Katagi, *Chromatographia*, 29 (1990) 587.
16 D.W. Armstrong, T.J. Ward, R.D. Armstrong and T.E. Beesley, *Science*, 232 (1986) 1132.
17 E. Kusters and D. Giron, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 9 (1986) 531.
18 E. Delee, L. Le Garrec, I. Jullien, S. Berranger, J.C. Pascal and H. Pinhas, *Chromatographia*, 24 (1987) 357.
19 J.A. Thompson, J.L. Holtzman, M. Tsuru, C.L. Lerman and J.L. Holtzman, *J. Chromatogr.*, 238 (1982) 470.
20 M.J. Wilson and T. Walle, *J. Chromatogr.*, 310 (1984) 424.
21 J. Gal and A.J. Sedman, *J. Chromatogr.*, 314 (1984) 275.
22 A.A. Gulaid, G.W. Houghton and A.R. Boobis, *J. Chromatogr.*, 318 (1985) 393.
23 P. Hsyu and K.M. Giacomni, *J. Pharm. Sci.*, 75 (1986) 601.
24 G. Pflugmann, H. Spahn and E. Mutschler, *J. Chromatogr.*, 421 (1987) 161.
25 S.K. Chin, A.C. Hui and K.M. Giacomini, *J. Chromatogr.*, 489 (1989) 438.
26 H.G. Schaefer, H. Spahn, L.M. Lopez and H. Derendorf, *J. Chromatogr.*, 527 (1990) 351.
27 G. Gübitz and S. Mihellyes, *J. Chromatogr.*, 314 (1984) 462.
28 A. Darmon and J.P. Thenot, *J. Chromatogr.*, 374 (1986) 321.
29 A.J. Sedman and J. Gal, *J. Chromatogr.*, 278 (1983) 199.

30 D. Schuster, M. Woodruff Modi, D. Lalka and F.M. Gengo, *J. Chromatogr.*, 433 (1988) 318.
31 E.J. Eisenberg, W.R. Patterson and G.C. Kahn, *J. Chromatogr.*, 493 (1989) 105.
32 R. Hasegawa, M. Murai-Kushiya, T. Komuro and T. Kimura, *J. Chromatogr.*, 494 (1989) 381.
33 J. Hermansson and C. von Bahr, *J. Chromatogr.*, 221 (1980) 109.
34 W. Lindner, C. Leitner and G. Uray, *J. Chromatogr.*, 316 (1984) 605.
35 W. Lindner, M. Rath, K. Stoschitzky and G. Uray, *J. Chromatogr.*, 487 (1989) 375.
36 J. Hermansson and C. von Bahr, *J. Chromatogr.*, 227 (1982) 113.
37 R.J. Guttendorf, H.B. Kostenbauer and P.J. Wedlund, *J. Chromatogr.*, 489 (1989) 333.
38 H.F. Schitthenner, M. Fedorchuk and D.J. Walter, *J. Chromatogr.*, 487 (1989) 197.
39 R. Mehvar, *J. Chromatogr.*, 493 (1989) 402.
40 R. Mehvar, *J. Pharm. Sci.*, 78 (1989) 1035.
41 S. Einarsson and B. Josefsson, *Anal. Chem.*, 59 (1987) 1191.
42 M. Ahnoff, S. Chen, A. Green and I. Grundevik, *J. Chromatogr.*, 506 (1990) 593.
43 W. Dieterle and J.W. Faigle, *J. Chromatogr.*, 259 (1983) 311.
44 M. Piquette-Miller, R.T. Foster, F.M. Pasutto and F. Jamali, *J. Chromatogr.*, 526 (1990) 129.
45 M. Piquette-Miller and R.T. Foster, *J. Chromatogr.*, 533 (1990) 300.
46 C. Petterson and G. Schill, *J. Chromatogr.*, 204 (1981) 179.
47 C. Petterson and M. Josefsson, *Chromatographia*, 21 (1986) 321.
48 A. Karlsson, C. Pettersson and B. Björkman, *J. Chromatogr.*, 494 (1989) 157.
49 C.L. Davies, *J. Chromatogr.*, 531 (1990) 131.
50 N. Nimura, H. Ogura and T. Kinoshita, *J. Chromatogr.*, 202 (1980) 375.
51 T. Kinoshita, Y Kasahara and N. Nimura, *J. Chromatogr.*, 210 (1981) 77.
52 N. Nimura, Y. Kasahara and T. Kinoshita, *J. Chromatogr.*, 213 (1981) 327.
53 P. Helboe, unpublished results.
54 *Analytical Validation. The rules governing medicinal products in the European Community*, Vol. III, Addendum 1, Brussels, 1990.