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Enantioseparation of β-blockers labelled with a chiral fluorescent reagent, R(−)-DBD-PyNCS, by reversed-phase liquid chromatography[1]

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Abstract

A fluorescent chiral tagging reagent, 4-(3-isothiocyanatopyrrolidin-1-yl)-7-(*N*,*N*-dimethylaminosulfonyl)-2,1,3-benzoxadiazole [R(−)-DBD-PyNCS], has been used for the liquid chromatographic resolution of racemic pairs of β-blockers. The reagent reacts with β-blockers at 65°C for 90 min in aqueous acetonitrile containing 0.05% triethylamine to produce the corresponding pair of diastereomers. No racemization occurs during the tagging reaction under these conditions. From results of the time-course study of oxprenolol the reactivities of the enantiomers of β-blockers with R(−)-DBD-PyNCS are comparable. The optimum excitation and emission wavelengths of the resulting derivatives were ca. 460 and 550 nm, respectively. The derivatives of β-blockers were efficiently resolved by a reversed-phase column with water-acetonitrile containing 0.1% trifluoroacetic acid as the eluent. The resolution (Rs) values of the diastereomers derived from 10 β-blockers were in the range of 1.54–4.80. The Rs value for timolol was 0.643. The detection limits (signal-to-noise ratio of 2) were one or two orders of magnitude lower with β-blockers having the iso-propylamino structure (15–300 fmol) than with those having the *tert*-butylamino structure (1.25–8.00 pmol). The proposed procedure was applied to the determination of R(+)- and S(−)-propranolol in rat plasma and saliva after oral administration of R(+)-propranolol hydrochloride or S(−)-propranolol hydrochloride. © 1997 Elsevier Science B.V.

Keywords: Fluorescent chiral tagging reagent; Pre-column derivatization; Resolution of enantiomers of β-blockers; Fluorescence detection; Reversed-phase liquid chromatography

1. Introduction

Many of the chiral drugs developed from organic syntheses are marketed as racemates [1]. However, it is known that enantiomers can have different activities, toxicities, pharmacokinetics and pharmacodynamic properties because the biological system has inherent chiral selectivity due

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to enzymes and receptors [2]. β-Blockers are well known as chiral drugs because of the asymmetric carbon in the structure. In some cases the enantiomers exhibit differences in activity [3,4]. For example, S(−)-propranolol is 60–100 times more potent as a β-blocker than the R(+)-enantiomer. Therefore, the enantioseparation of β-blockers is an important aspect of quality control, metabolic studies, blood level studies, etc.

Highly sensitive detection is required for trace analysis of chiral drugs in biological samples. Gas chromatography (GC) [5,6], liquid chromatography (LC) [7,8] and capillary electrophoresis (CE) [9,10] have been reported for the separation of chiral molecules. Of these techniques, LC is often the method of choice because of ease of handling and sample treatment, as well as excellent reproducibility, accuracy and precision. Techniques for the resolution of racemates are categorized as: the direct method using a chiral stationary phase (CSP) column; or the indirect method involving derivatization with a chiral reagent. In cases requiring sensitive determination in biological samples such as blood and urine, the indirect method is recommended because various chiral reagents with properties that contribute to a highly sensitive detector response can be used for diastereomer formation. There are many ultraviolet-visible (UV-VIS) and fluorescence (FL) labels for the derivatization of β-blockers, e.g. orthophthalaldehyde (OPA)/chiral thiol such as *N-tert*-butyloxycarbonyl (BOC)-*N*-acetyl-L-cysteine or *N*-acetyl-D-penicillamine [11] and chiral 1-(9-fluorenyl)ethyl chloroformate (FLEC) [12,13]. These reagents have been successfully applied to the determination of various β-blockers in biological samples [14–17].

In a previous report [18], the synthesis was described of novel fluorescent chiral tagging reagents for the amino functional group, S(+) and R(−) enantiomers of 4-(3-isothiocyanatopyrrolidin-1-yl)-7-nitro-2,1,3-benzoxadiazole (NBD-PyNCS) and 4-(3-isothiocyanatopyrrolidin-1-yl)-7-(*N*,*N*-dimethylaminosulfonyl)-2,1,3-benzoxadiazole (DBD-PyNCS). These reagents have been successfully applied to the enantioseparation of various amines including amino acids [19–21]. This paper describes the resolution of racemic β-blockers after derivatization with R(−)-DBD-PyNCS by liquid chromatography. The applicability of the proposed procedure is shown by the determination of R(+)- and S(−)-propranolol in plasma and saliva of the rat after oral administration.

2. Experimental

2.1. Materials and reagents

4-(3-Isothiocyanatopyrrolidin-1-yl)-7-(*N*,*N*-dimethylaminosulfonyl)-2,1,3-benzoxadiazole [R(−)-DBD-PyNCS] was synthesized as described previously [18]. Both enantiomers of propranolol hydrochloride (HCl) were purchased from Sigma (St. Louis, MO, USA). Racemic mixtures of atenolol, pindolol, alprenolol HCl, bucumolol HCl, bupranolol HCl, carteolol HCl, indenolol HCl, oxprenolol HCl, and timolol HCl were gifts from the National Institute of Health Sciences (NIHS, Tokyo, Japan). The structures of β-blockers used are depicted in Fig. 1. Sodium pentobarbitone (Nethanbutol injection) was obtained from Dainippon Pharmaceutical (Tokyo, Japan). Pilocarpine HCl, urethane (carbamic acid ethyl ester) and triethylamine (TEA) were special reagent grade from Wako Pure Chemicals (Osaka, Japan). Trifluoroacetic acid (TFA), acetonitrile (CH_3CN) and ethyl acetate (AcOEt) were of HPLC grade (Wako). De-ionized and distilled water was used throughout. All other chemicals were of analytical-reagent grade and were used without further purification.

2.2. HPLC

The high-performance liquid chromatograph consisted of a CCPM pump and a PX-8010 controller (Tosoh, Tokyo, Japan). A Rheodyne 7125 injector (Cotati, CA, USA) was used. The 150 × 4.6 mm i.d. column was packed with 5 μm Inertsil ODS-80A (GL Sciences, Tokyo, Japan). The column was maintained at 40°C with a CO-8020 column oven (Tosoh). A Hitachi L-7480 fluorescence monitor equipped with a 12-μl flow cell (Tokyo, Japan) was used for the detection of the

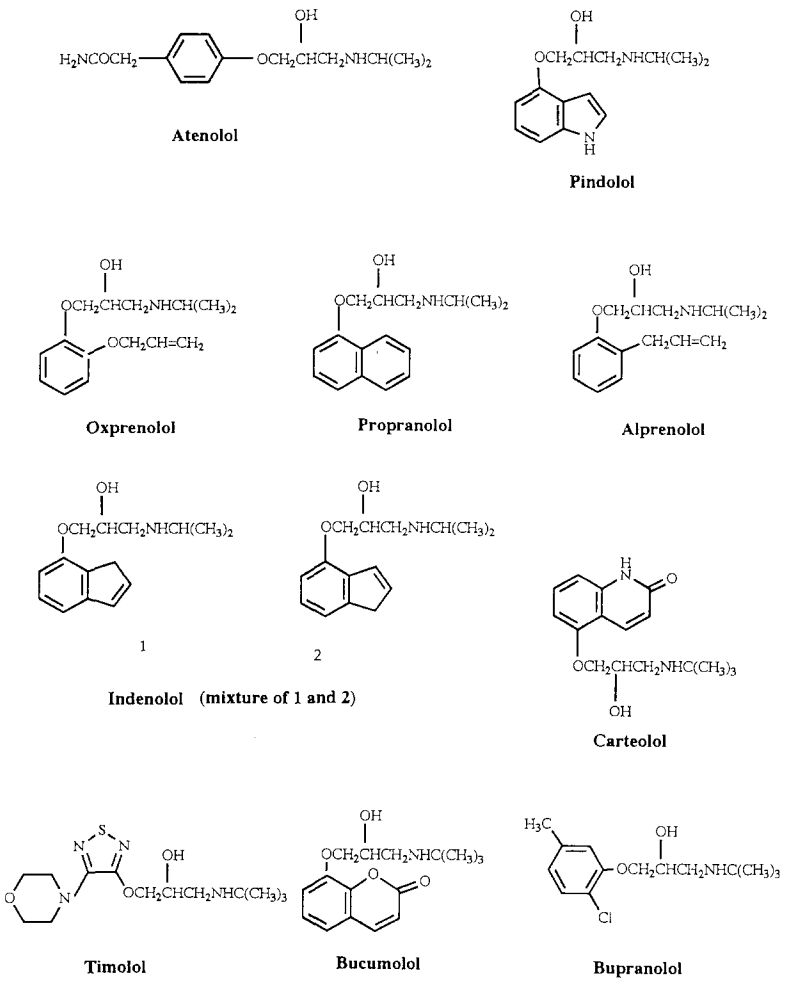
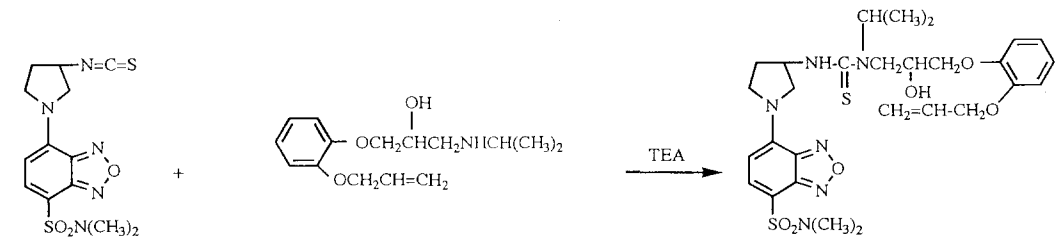


Fig. 1. Structures of β-blockers tested.

derivatives. The excitation and emission wavelengths were fixed at 460 and 550 nm, respectively. The peak areas obtained from the fluorescence monitor were calculated with a C-R4A Chromatopac (Shimadzu). The eluent consisted of various concentrations of aqueous acetonitrile solution containing 0.1% of TFA. All mobile phases were de-gassed with an on-line degasser (SD-8022, Tosoh). The flow-rate of the eluent was 1 ml min^{-1}.

2.3. Animals

Wistar/ST male rats (12–14 weeks old; 320–410 g) were supplied by Shizuoka Laboratory Animal Center (Hamamatsu, Japan). The rats were housed in cages under standard laboratory conditions (room temperature, 23 ± 1°C; relative humidity, 55 ± 5%; light/dark cycle, 12 h/12 h) and were given a commercial pellet diet (Oriental-MF, Tokyo, Japan) and water ad libitum.



R(-)DBD-PyNCS Oxprenolol

Fig. 2. Reaction of oxprenolol with R(−)-DBD-PyNCS.

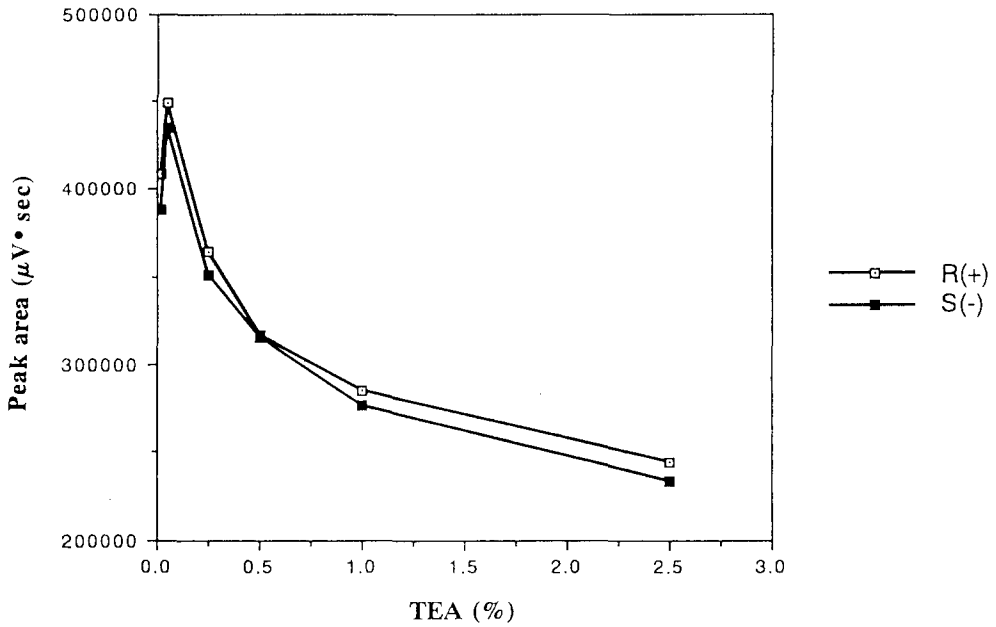


Fig. 3. Effect of TEA concentration on the derivatization of oxprenolol with R(−)-DBD-PyNCS.

2.4. Derivatization procedure for β-blockers

A 100-μl portion of 1 mM R(−)-DBD-PyNCS in acetonitrile and a 100 μl aliquot of the racemic β-blocker (about 10 μM) in acetonitrile-water (1:1, v/v) containing 0.1% triethylamine (TEA) were mixed in a 1.5 ml mini-vial (GL Science). The vials were tightly capped and heated at 65°C with a dry heat block for 90 min. Then an aliquot of the mixture was injected into the column. The reagent blank without β-blockers was also treated in the same manner.

The capacity factor (k'), separation factor (α) and the resolution value (Rs) were calculated according to the following equations:

$$k' = (t_R - t_0)/t_0, \qquad \alpha = k'_2/k'_1,$$

$$Rs = 2(t_{R2} - t_{R1})/(w_1 + w_2)$$

where t_R, t_{R1} and t_{R2} are the peak retention times, t_0 is due to the void volume of the column ($t_0 = 1.4$ min) and w_1 and w_2 are the widths of the bases formed by triangulation of the peaks.

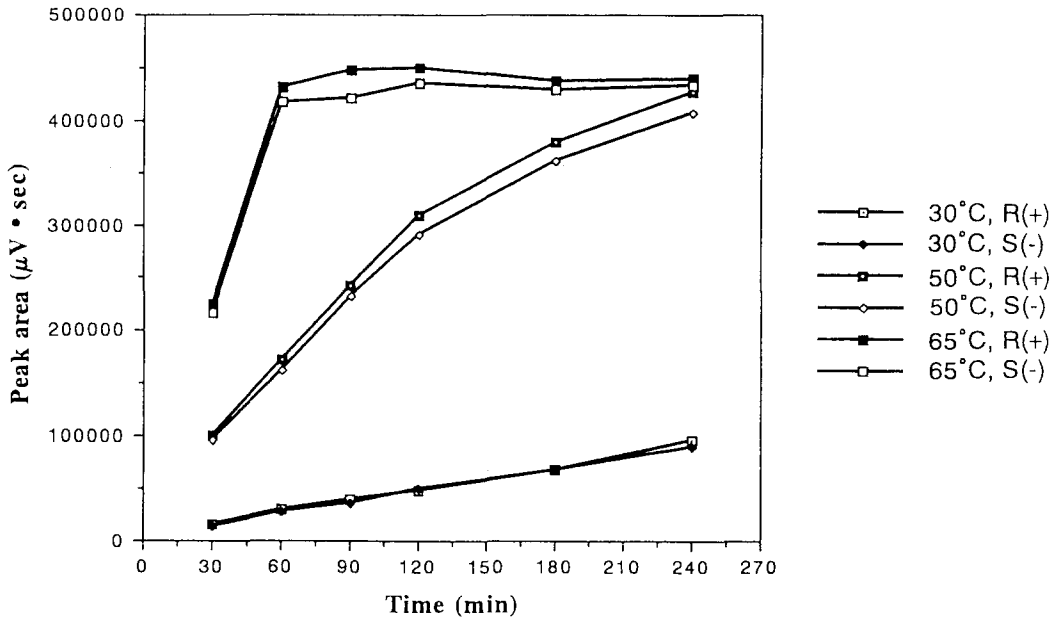


Fig. 4. Effect of temperature on the derivatization reaction of oxprenolol with R(−)-DBD-PyNCS.

2.5. Sample treatment and determination of R(+)- and S(−)-propranolol in rat plasma and saliva

The rats were fasted for 18 h prior to the experiments and anaesthetized with pentobarbitone (50 mg kg^{-1}, i.p.) and urethane (0.75 g kg^{-1}, s.c.) to take blood and saliva samples. After tracheotomy and catheterization, the femoral vein was cannulated with polyethylene tubing for infusion of pilocarpine hydrochloride at a constant rate of 3.0 mg kg^{-1} h^{-1} to stimulate salivation [22]. After 2 h of infusion of pilocarpine, R(+)- or S(−)-propranolol was administered as a bolus dose equivalent to 10 mg kg^{-1}. The jugular vein was also cannulated with silicon polymer tubing for collection of blood samples. The mandibular gland was exposed carefully and bevelled polyethylene tubing (PE-10, Clay Adams, Tokyo, Japan) was inserted into the duct to collect saliva samples. Saliva samples were collected periodically for 30 min; blood samples were withdrawn at the endpoint of the saliva collection intervals. The plasma samples were separated by centrifugation (3000 rpm for 10 min) of the heparinized blood.

To 50 μl of plasma or saliva was added 350 μl of internal standard (IS) solution in water (1 μM oxprenolol hydrochloride). After addition of 100 μl of NaOH solution (4 M), propranolol and internal standard (oxprenolol) in the alkaline solution were thoroughly mixed with sonication for 10 min and then extracted with 3 ml of ethyl acetate. The organic layer separated after centrifugation (3000 rpm for 3 min) was washed with saturated sodium chloride (NaCl) and dried with anhydrous sodium sulfate (Na_2SO_4). The ethyl acetate extract (2.0 ml) was transfered to a glass tube and evaporated to dryness in vacuo. The residue obtained from plasma or saliva was dissolved in 100 μl of 50% (v/v) acetonitrile-water containing 0.1% TEA, and then reacted with 100 μl of 1 mM R(−)-DBD-PyNCS at 65°C for 90 min in the dark. After derivatization, an aliquot of the reaction solution (50 μl) was injected onto the column for HPLC. The eluent was acetonitrile-water (56:44, v/v) containing 0.1% TFA. The concentrations of R(+)- and S(−)-propranolol in rat plasma and saliva were calculated from the calibration curves based on the area ratio with the internal standard.

Table 1
Separation and detection of β-blockers labelled with R(−)-DBD-PyNCS

Drug	CH_3CN/H_2O	Retention time (min)	k'	α	Rs	Detection limit (fmol)
Atenolol	3.5/6.5	26.7	18.1	1.24	3.9	96
		32.8	22.4			116
Pindolol	4.4/5.6	30.9	21.1	1.29	4.1	296
		39.4	27.1			320
Oxprenolol	6.2/3.8	10.2	6.26	1.21	2.5	15
		12.0	7.59			16
Propranolol	6.2/3.8	11.4	7.13	1.28	3.5	25
		14.1	9.10			29
Alprenolol	6.2/3.8	12.4	7.83	1.32	4.1	31
		15.9	10.3			39
Indenolol	5.5/4/5	(1)21.6	14.5	1.29	4.4	91
		27.7	18.8			105
		(2)20.6	13.7	1.29	4.3	182
		26.1	17.7			222
Carteolol	3.5/6.5	23.4	15.7	1.22	3.5	6.35×10^3
		28.3	19.2			8.00×10^3
Timolol	3.5/6.5	28.8	19.6	1.04	0.64	5.95×10^3
		29.9	20.4			6.42×10^3
Bucumolol	3.7/6.3	37.2	25.5	1.08	1.5	3.03×10^3
		40.0	27.6			3.28×10^3
Bupranolol	5.0/5.0	29.4	20.0	1.25	4.8	1.25×10^3
		36.5	25.1			1.61×10^3

3. Results and discussion

3.1. Optimization of the derivatization

Fig. 2 shows the derivatization reaction of oxprenolol with DBD-PyNCS. The reaction proceeds in an alkaline medium to produce the corresponding diastereomer. Pyridine, TEA, 1,8-diazabicyclo[5.4.0]-7-undecence (DBU) and quinuclidine are equally effective as catalysts for the derivatization [19]. In this research, TEA was selected for the derivatization because of its ready availability. With respect to the concentration, the highest peak derived from oxprenolol, selected as a representative β-blockers, was obtained with a 0.05% TEA solution; higher concentrations than 0.25% reduced the yield of the derivatives. Therefore, 0.05% TEA was selected in the following experiment (Fig. 3). Reaction temperature is another important factor for the tagging reaction. Time courses of the reaction were tested at 30, 50 and 65°C in the presence of 0.05% TEA. As shown in Fig. 4, higher temperatures gave faster reaction rates and higher yields of the derivative. The reactivity of R(−)-DBD-PyNCS does not favour one enantiomer over the other. The reaction curves derived from the optical isomers of oxprenolol were essentially superimposable. The results suggest that a reaction time of more than 60 min at 65°C is suitable for quantitative derivatization of β-blockers. Thus, the reaction conditions of 65°C for 90 min in the presence of 0.05% TEA was selected for the derivatization of β-blockers with R(−)-DBD-PyNCS.

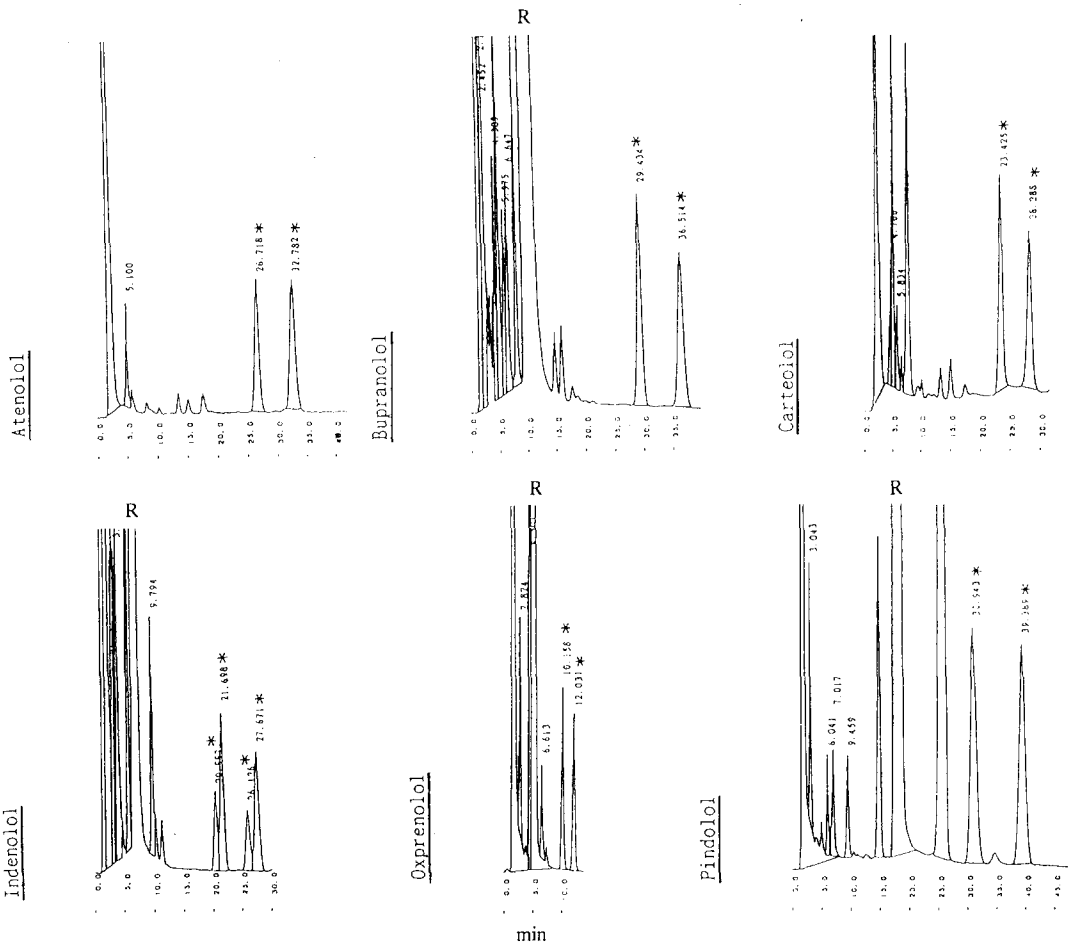


Fig. 5. Typical chromatograms of the derivatives of β-blockers obtained from R(−)-DBD-PyNCS by isocratic elution. Asterisked peaks show the pairs of diastereomers. R, R(−)-DBD-PyNCS. All mobile phases contain 0.1% TFA. Other HPLC conditions are given in the Experimental section.

3.2. Separation and detection of β-blockers labelled with R(−)-DBD-PyNCS

Chromatographic separations of FL derivatives of β-blockers were carried out with isocratic elution on a reversed-phase column. The capacity factors (k'), separation factors (α), resolution values (Rs), and detection limits (signal-to-noise ratio of 2) for each pair of the derivatives are shown in Table 1. The derivatives, except timolol, were completely separated with a water-acetonitrile mixture containing 0.1% TFA as the eluent. Fig. 5 shows some typical chromatograms obtained from reaction solutions of racemic β-blockers with R(−)-DBD-PyNCS. Although each pair of β-blockers was completely separated by isocratic elution, simultaneous analysis on a single chromatographic run is really difficult owing to unknown interfering peaks. β-Blockers with the iso-propylamino moiety (i.e. oxprenol, propranolol, alprenolol, atenolol, indenolol and pindolol; 16–320 fmol) can be detected at lower levels than β-blockers with the *tert*-butylamino moiety (i.e. bupranolol, bucmolol, carteolol, timolol; 1.25–8.0 pmol) (Table 1). The lower sensitivity may be due to low derivatization yield because of steric hindrance of the reaction site of the β-blockers. Consequently, for maximum sensitiv-

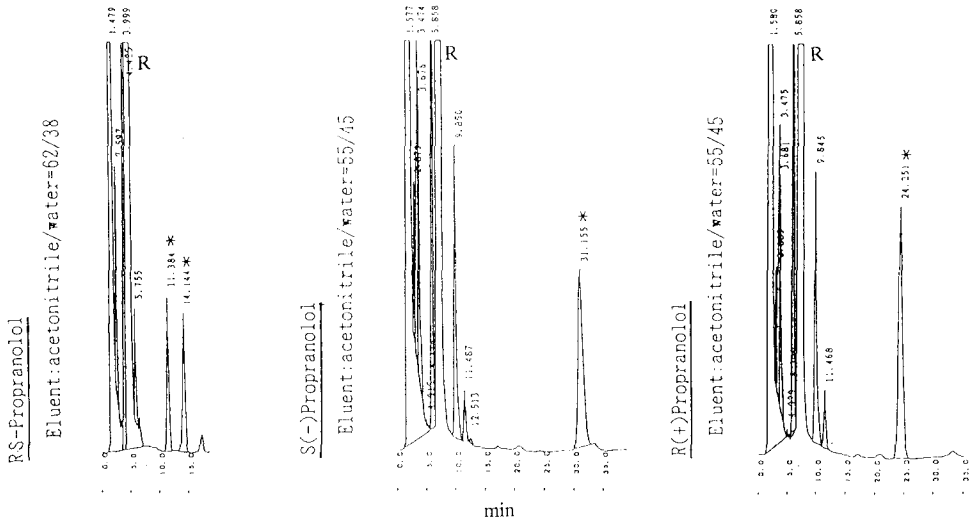


Fig. 6. Chromatograms of R(+)- and S(−)-propranolols labelled with R(−)-DBD-PyNCS. Asterisk peaks show the pairs of diastereomers. R, R(−)-DBD-PyNCS. All mobile phases are containing 0.1% TFA. Other HPLC conditions are given in the Experimental section.

ity, the proposed procedure should be limited to those β-blockers possessing an iso-propylamino structure.

3.3. Determination of R(+)- and S(−)-propranolol in rat plasma and saliva

Since racemization during the derivatization reaction is an important factor for trace analysis of each enantiomer, R(+)-propranolol or S(−)-propranolol was separately derivatized with R(−)-DBD-PyNCS by the proposed procedure. As shown in Fig. 6, the peak corresponding to the opposite enantiomer was not observed in these chromatograms.

Linear relationships (correlation coefficient, $r > 0.996$) of the calibration curves were obtained from the comparison between the concentration of R(+)- or S(−)-propranolol and the peak-area ratio of the I.S. (oxprenolol) by the recommended procedure. The equations of the linear curves were: $y = 1.077 \times -0.0075$ for R(+)-propranolol; and $y = 1.141 \times -0.0143$ for S(−)-propranolol. A slight difference of the slope was observed in both enantiomers (1.077 vs. 1.141). However, it seems to present no problem in the determination of propranolol enantiomers in biological samples. The above results prompted the use of the proposed procedure for the determination of the racemic pair of propranolol in real samples [23,24].

As one application of the chiral tagging reagent DBD-PyNCS, the concentrations of R(+) and S(−)-propranolol in rat plasma and saliva were determined after oral administration equivalent to 10 mg kg^{-1}. When R(+)-propranolol was administrated to rats, only the R(+)-enantiomer appeared in the plasma and saliva. The concentration was much higher in plasma than in saliva (Fig. 7A). In the administration of S(−)-propranolol, on the other hand, the opposite was true. Furthermore, the R(+)-enantiomer was also identified in the plasma and saliva samples at high concentrations. The concentration was about one-order of magnitude higher in saliva than in plasma at all sampling times; in contrast, the concentration of S(−)-propranolol in saliva was only a few times higher (Fig. 7B). Since racemization during the derivatization reaction is negligible, as shown in Fig. 6, the production of the R(+)-enantiomer must be due to internal conversion of S(−)-propranolol [2]. Although such

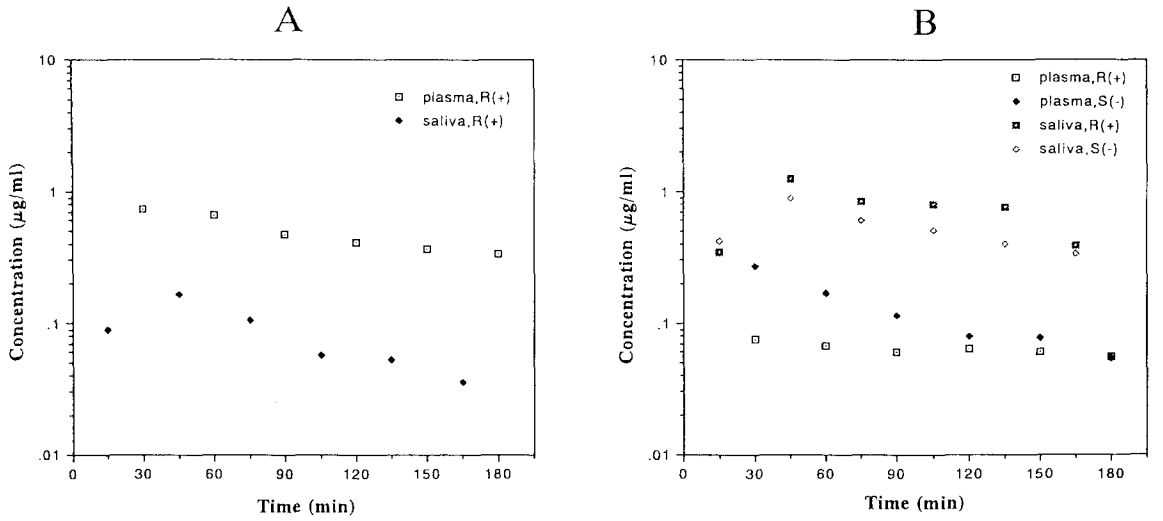


Fig. 7. Concentration of propranolols in rat plasma and saliva after oral administration (10 mg kg^{-1}). A, Administration of R(+)-propranolol hydrochloride; B, Administration of S(−)-propranolol hydrochloride.

metabolic inversion has been shown in the administration of ibuprofen and related anti-inflammatory drugs [25], there have been no reports about β-blockers. The mechanism of the conversion is not clear but should be solved with further studies. A comparison study of the concentration in plasma and saliva is currently in progress.

4. Conclusions

The chiral separation of primary and secondary amines involving β-blockers with the indirect fluorescence derivatization by liquid chromatography has mainly been performed by the methods using OPA/chiral thiol, 1-(1-naphthyl)ethyl isocyanate (NEIC) [15,16] and FLEC. Although OPA/chiral thiol reacts with primary amines under mild conditions, secondary amines such as β-blockers do not react with the reagent owing to the lack of a primary amino group. The derivatization conditions (i.e. high temperature and long time) of amines with NEIC possessing an isocyanate group as a reactive site seem to be rather rigorous for real sample analysis. On the other hand, for the enantiomer of FLEC (+ or − form), the derivatization is excellent in terms of reaction conditions, chromatographic separation and detection sensitivity. However, the wavelengths of the resulting derivative with FLEC are in the short region (i.e. excitation at about 260 nm; emission at about 310 nm), similar to those with OPA chiral thiol (ex. 350 nm; em. 445 nm) and NEIC (ex. 285 nm; em. 330 nm).

β-Blockers possessing the iso-propylamino moiety react quantitatively with DBD-PyNCS to produce corresponding the diastereomers which fluoresce in the long wavelength region (ex. 460 nm; em. 550 nm). The characteristics of the wavelengths are distinct advantage of DBD-PyNCS. The resulting derivatives are easily resolved by reversed-phase chromatography. The limits of detection are at the femtomole levels with the conventional fluorescence detector and comparable to those with FLEC and OPA/chiral thiol. However, the sensitivity may be improved with chemiluminescence detection because the structure of DBD-amine strongly fluoresces with the reaction of the oxalate ester derivative and hydroxyperoxide [26–28]. Hence, the proposed method using DBD-PyNCS enantiomer may serve for enantiomeric quantification of chiral drugs in real samples.

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