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Short Communication

Reversed-phase high-performance liquid chromatographic separation of the stereoisomers of labetalol via derivatization with chiral and non-chiral isothiocyanate reagents

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ABSTRACT

The antihypertensive agent labetalol is a mixture of two racemates. We report reversed-phase high-performance liquid chromatographic (HPLC) methodology for the separation of the four stereoisomers of labetolol via derivatization with the chiral reagent (4*S*-*cis*)-2,2-dimethyl-5-isothiocyanato-4-phenyl-1,3-dioxane. The derivatives were separated on octadecylsilane columns with a methanol–ammonium phosphate buffer mixture as mobile phase. Separations of the diastereomeric forms of labetalol were achieved with the non-chiral derivatizing reagents benzyl isothiocyanate and 1-naphthalenemethyl isothiocyanate. In all cases the derivatives of the *R,S*/*S,R* forms eluted before those of the *R,R*/*S,S* forms. Isothiocyanates may have general utility in stereoisomer separations of amines by HPLC.

INTRODUCTION

Labetalol is a widely used antihypertensive drug with α- and β-adrenergic antagonistic and other pharmacological effects [1,2]. The chemical structure (Fig. 1) of labetalol contains two stereogenic centers, allowing the existence of four stereoisomers that can form two racemates. In one of the racemates the two enantiomers have the *R,R* and *S,S* configuration, respectively, and the enantiomers of the other racemate are of the *R,S* and *S,R* configuration, respectively. Any two of the four stereoisomers that are not enantiomerically related (*i.e.* are not mirror images of

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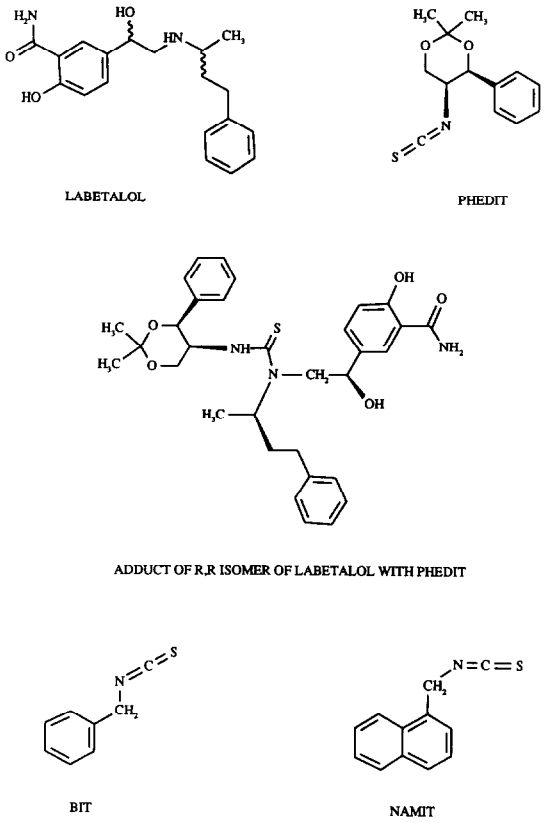

Fig. 1. Chemical structures of the compounds studied. The wavy bonds at the two stereogenic centers of labetalol indicate the presence of both configurations at each center, *i.e.*, the presence of four stereoisomers.

each other) are diastereoisomers (epimers). The clinically used drug is a mixture of the four stereoisomers in the form of an approximately equimolar mixture of the two racemates [3]. The pharmacological properties of the four stereoisomers of labetalol, however, differ drastically [3,4]. Furthermore, evidence has been presented by us [5] and by others [6,7] that labetalol may be subject to stereoselective metabolism. It is important, therefore, that studies of the disposition of labetalol should be carried out stereospecifically, if we are to understand the role of each of the stereoisomers in the effects of the drug. Prerequisite to such studies, however, is the availability of stereospecific analytical methods that allow the determination of the concentration of each stereoisomer in the presence of the others. Chromatography is eminently suitable for the stereospecific analysis of drugs in biological fluids and is widely used for this purpose.

Several reports have described the chromatographic separation of the diastereomers of labetalol using non-chiral chromatographic conditions [7–10]; in these procedures each racemate gave a single peak, because enantiomerically related stereoisomers cannot be separated via these techniques. Such methods include the separation of the racemates of derivatized [7,8] or underivatized [9,10] labetalol, and used gas chromatography [7,8], high-performance liquid chromatography (HPLC) [9], or thin-layer chromatography [10]. Some of these methods have been applied to the analysis of the racemate ratio in pharmaceutical dosage forms of labetalol [9], and to the determination of the ratio of the diastereomers of labetalol excreted in the urine of volunteers administered oral labetalol [7]. Concerning the latter application, it should be noted that *in vivo* concentrations of two enantiomers can differ from the racemic, 1:1, ratio administered, but the analytical method used provided only the sum of the two enantiomers of each racemate [7].

Lindner *et al.* [11] described the derivatization of several alkanolamines, including labetalol, with optically active tartaric acid monoesters and separation of the resulting derivatives by reversed-phase HPLC. While this method could in principle separate the four stereoisomers, apparently only two peaks were obtained for labetalol, and the authors did not comment on the identity of the stereoisomers giving rise to the two peaks [11].

Little has been published on the chromatographic separation of all four stereoisomers of labetalol. Schill *et al.* [12] reported that a chiral HPLC column based on α_1-acid glycoprotein was capable of resolving each of the two racemates of labetalol into its enantiomers, but the authors did not comment on the separation of all four isomers in a single run, and the information provided is insufficient to determine the extent of separations achieved. More recently, Lalonde *et al.* [13] attempted to determine the concentration of

the four stereoisomers in human blood plasma using the α_1-acid glycoprotein chiral HPLC column, but data on the resolutions, *e.g.* the separation factors α and the resolution factors, were not reported, nor were any chromatograms shown, and thus the nature and extent of the separations are not known. The assay was not sufficiently sensitive to permit a full pharmacokinetic study of all four isomers and only the results from the analysis of the samples of highest concentrations were reported [13].

It is clear that additional chromatographic methods that can separate the stereoisomers of labetalol would be of interest. We report here HPLC separation methods using derivatizations of the drug with chiral and non-chiral reagents based on the reaction of the isothiocyanate moiety of the reagent with the secondary amino functionality of labetalol.

EXPERIMENTAL

Materials

(4*S*,5*S*)-(+)-5-Amino-2,2-dimethyl-4-phenyl-1,3-dioxane (ADPD) was obtained from Sigma (St. Louis, MO, USA). Benzyl isothiocyanate 98%, 1,1′-thiocarbonyldiimidazole (TCDI) and triethylamine 99+% were purchased from Aldrich (Milwaukee, WI, USA); 1-naphthalenemethyl isothiocyanate was obtained from Trans World Chemicals (Chevy Chase, MD, USA). HPLC-grade acetonitrile, dichloromethane and methanol were obtained from Burdick and Jackson (Muskegon, MI, USA). Ammonium phosphate, monobasic, HPLC grade, was purchased from J. T. Baker (Phillipsburg, NJ, USA). Labetalol, its *R*,*R* and *S*,*S* forms, and the *S*,*R*/*R*,*S* racemate were a gift from Dr. John Thompson, School of Pharmacy, University of Colorado.

(4*S*-*cis*)-2,2-Dimethyl-5-isothiocyanato-4-phenyl-1,3-dioxane was synthesized as described in a preliminary communication [14]. In a 500-ml round-bottomed flask was placed 150 ml of dichloromethane containing TCDI (5.0 g; 0.028 mol). ADPD (5.0 g, 0.04 mol) dissolved in 150 ml of dichloromethane was added dropwise with vigorous magnetic stirring. The reaction was allowed to proceed at room temperature for 3 h with continuous stirring. The contents of the flask were then poured into a separatory funnel and washed three times with 300-ml portions of 5% sodium bicarbonate, followed by three 300-ml portions of water. The organic layer was then dried over anhydrous sodium sulfate for 20 min, filtered and the solvent removed *in vacuo* on a rotary evaporator. The pale yellow solid obtained was purified by recrystallization from absolute ethanol. The product, obtained in 89% yield, had a melting point of 105–106°C. Its elemental analysis, proton nuclear magnetic resonance and electron-impact mass spectra were consistent with the expected structure.

Derivatizations

Labetalol or one of the isomeric forms, as the hydrochloride (1 mg), was placed in a conical centrifuge tube and treated with 3 mg of one of the derivatizing agents. Acetonitrile (100 μl), water (50 μl), and triethylamine (3 μl) were added. The tube was capped with a rubber stopper and swirl-mixed (vortex); derivatization was then allowed to take place for 1 h at 60°C. At the end of the derivatization period the tube was allowed to cool to room temperature, and an aliquot of 150 μl was then removed and diluted with 400 μl of acetonitrile. The tube was swirl-mixed and aliquots of 5 μl were injected into the liquid chromatographic system.

Chromatography

A Waters Assoc. (Milford, MA, USA) HPLC system consisting of a Model U6K injector, a Model 6000A solvent delivery system and a Model 480 Lambda Max LC spectrophotometer were used. The separations were carried out on a Waters Assoc. Nova-Pak C_{18} column, 150 mm × 3.9 mm I.D., with 4 μm particle size. The column effluent was monitored at 254 nm, and the detector output was recorded using a Hewlett Packard 3390A electronic integrator. The buffer employed in the mobile phases was 0.02 *M* ammonium phosphate, monobasic, pH 4.60. The mobile phases were mixtures of methanol and buffer in the proportions indicated in Figs. 2 and 3. The mobile phase was delivered at 1 ml/min.

RESULTS AND DISCUSSION

Isothiocyanates react with primary or secondary amines to form the corresponding thiourea derivatives; if the isothiocyanate reagent is chiral and is used in the form of a single enantiomer to derivatize a racemic amine, two diastereomeric thiourea derivatives are formed which may be separable on traditional (non-chiral) chromatographic columns [15]. This approach to chromatographic chiral separation is often termed the indirect approach inasmuch as the separation of diastereomeric derivatives rather than of the enantiomers *per se* (the direct method), is carried out. The indirect method, although conceptually inferior to the direct method, is popular for a variety of reasons and has been used to solve a variety of qualitative and quantitative stereochemical problems [15]. The isothiocyanate moiety is well suited for the role of derivatization of the amino group: its reaction with amines is rapid and clean; the derivatization is selective as hydroxyl and carboxyl groups do not react under conditions sufficient for the derivatization of the amino group; the thiourea moiety imparts significant ultraviolet light-absorbing properties to the derivatives; the reaction of the isothiocyanate moiety with water is sufficiently slow that the reaction with target amines can be conducted in aqueous solution or in aqueous–organic mixtures, a great advantage when the derivatization of polar amines such as amino acids [16] or catecholamines [17] is considered. Furthermore, isothiocyanates are generally stable chemically and stereochemically; indeed, their chemical stability is an advantage over their isocyanate analogues, some of which have been used as chiral derivatizing agents [18].

A variety of optically active isothiocyanate derivatizing agents have been evaluated in our laboratory for their ability to provide diastereomeric derivatives of chiral amines that are separable by HPLC, including β-D-glucopyranosyl isothiocyanate 2,3,4,6-tetraacetate [19], (*R*)-(1-isothiocyanatoethyl)benzene [20], (*S*)-1-(1-isothiocyanatoethyl)naphthalene [21], and (*R*)-2-(1-isothiocyanatoethyl)naphthalene [21]. Since labetalol consists of two racemates, a successful chiral derivatizing agent must provide four derivatives that are adequately separated under some suitable chromatographic conditions. None of the above-mentioned isothiocyanate reagents, however, gave adequate resolution of the stereoisomers of labetalol (data not shown). Alternative reagents were therefore sought, and one of the products of this search was (4*S*-*cis*)-2,2-dimethyl-5-isothiocyanato-4-phenyl-1,3-dioxane (PHEDIT), conveniently prepared from its commercially available and inexpensive resolved primary amine precursor [5,14]. The chemical structure (Fig. 1) of PHEDIT contains two stereogenic centers, both of which have the *S* configuration in the reagent. The enantiomeric purity of the reagent was found to be at least 99.5% as determined by derivatizing a sample (−)-ephedrine of known enantiomeric purity [22].

The reaction of labetalol with excess PHEDIT was carried out as usual for derivatization of amines with isothiocyanates [21]. Since the isothiocyanate moiety does not react with the hydroxyl group under the reaction conditions used [23], only the amino group of labetalol is derivatized. The chemical structure of the derivative of the *R,R* isomer of labetalol formed with PHEDIT is shown in Fig. 1 to illustrate the

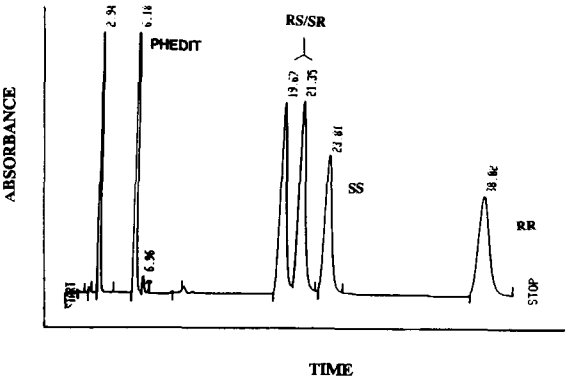

Fig. 2. Separation of the PHEDIT derivatives of labetalol; mobile phase: methanol–buffer (63:37, v/v). For derivatization and details of chromatographic conditions see Experimental. The identity of the isomers giving rise to the derivatives eluted is given; the assignment of the *R,S/S,R* isomers to the corresponding peaks was not possible, see text. Retention times: *R,S/S,R*: 19.67 and 21.35 min; *S,S*: 23.81 min; *R,R*: 30.02 min.

TABLE I

SEPARATIONS OF THE STEREOISOMERS OF LABETALOL USING DERIVATIZATIONS WITH ISOTHIOCYANATE REAGENTS

See Experimental for derivatization and chromatographic conditions.

Derivatizing reagent	Separation of					
	R,R from *S,S*		*S,R* from *R,S*		*S,R/R,S* from *R,R/S,S*	
	α^a	R^b	α	R	α	R
PHEDIT	1.62	8.55	1.09[c]	1.46	1.12[d]	1.85[d]
BIT	–[e]		–[e]		1.39	3.41
NAMIT	–[e]		–[e]		1.44	5.68

[a] Separation factor, see ref. 24, p. 35.
[b] Resolution factor, see ref. 24, p. 34
[c] Order of elution of the two derivatives unknown.
[d] Separation of the second peak of racemate *R,S/S,R* from the peak due to *S,S* isomer.
[e] Only separation of diastereomers occurs with the non-chiral reagents BIT and NAMIT.

chemistry of the derivatization reaction. Acetonitrile is a solvent of choice for derivatizations with isothiocyanates [19–21], suitably polar for the reaction, able to dissolve the reagents and many of the amines, and compatible with mobile phases used in reversed-phase HPLC. Furthermore, acetonitrile can also be used as a mixture with water, which may be necessary if the substrate amine is insufficiently soluble in organic solvents or if it is added in the form of one of its salts. In the latter case a basic catalyst must also be added in order to promote the reaction, and for this purpose we used triethylamine. The derivatization of labetalol with PHEDIT proceeded smoothly to produce four derivatives whose separation could be readily achieved under reversed-phase HPLC conditions on octadecylsilane columns (Fig. 2, Table I). Excess, unreacted PHEDIT had a short retention time and did not interfere with elution of the derivatives of labetalol (Fig. 2). In accordance with our previous findings on the derivatization of amines with isothiocyanates [19–21], the derivatives obtained produced good chromatographic band shapes under reversed-phase HPLC conditions. This is a useful feature of such derivatizations in general, inasmuch as the thiourea derivatives display much-improved chromatographic behavior over the underivatized amines.

Samples of the *R,R* and *S,S* isomers of labetalol were available, but only the racemic mixture of the *R,S/S,R* forms could be obtained. Using

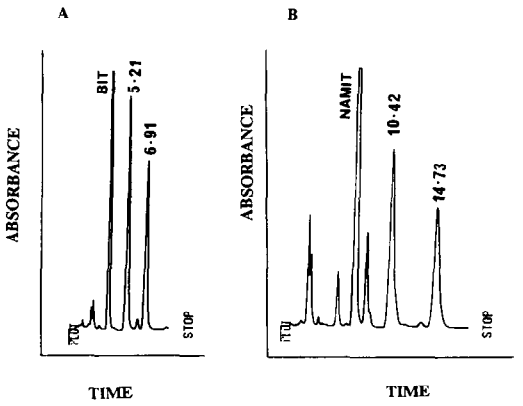

Fig. 3. Separations of the diastereomers of labetalol after derivatization with BIT (A) or NAMIT (B). Mobile phase: methanol–buffer (70:30, v/v). For derivatization and details of chromatographic conditions see Experimental. The retention time in minutes of each eluted derivative is shown. (A) Peak at 5.21 min: derivatives due to the *R,S/S,R* racemate; peak at 6.91 min: derivatives due to *S,S/R,R* racemate. (B) Peak at 10.42 min: derivatives due to the *R,S/S,R* racemate; peak at 14.73 min: derivatives due to *S,S/R,R* racemate.

these samples the order of elution of the derivatives could be assigned as shown in Fig. 2. The derivatives of the *R,R* and *S,S* stereoisomers were well separated; the resolution of the *R,S/S,R* racemate was less extensive, but was nearly "baseline", as indicated by the value of the resolution factor *R*, 1.46 (Table I); a value of 1.50 for this parameter means that the separation of two equal-sized chromatographic peaks is essentially complete (ref. 24, p. 35). The second peak of the *R,S/S,R* racemate was well separated from the peak due to the derivative of the *S,S* isomer (Fig. 2, Table I).

Our observation that PHEDIT, a chiral reagent, provided four diastereomeric derivatives of labetalol that were separable by reversed-phase HPLC suggested that derivatization with a non-chiral isothiocyanate may provide derivatives of the labetalol diastereomers separable by HPLC. As indicated above, such separations are of interest for the determination of the racemate ratio in pharmaceutical dosage forms. Furthermore, there may be a new application for such chromatographic separations of the diastereomers of labetalol in their analysis in biological fluids, since it was recently proposed [25] that, on the basis of the pharmacological properties of the stereoisomers of labetalol, the 50:50 mixture of the *R,R* and *S,R* (*S* at the hydroxyl-bearing stereogenic center) isomers may be a therapeutically useful combination, with potentially less toxicity than that of labetalol.

We previously found that derivatization of epimeric amines related to chloramphenicol with the non-chiral reagent isothiocyanatomethylbenzene (benzyl isothiocyanate, BIT, see chemical structure in Fig. 1) produced derivatives that were separable by reversed-phase HPLC [26]. BIT and the related reagent 1-(isothiocyanatomethyl)naphthalene(1-naphthalenemethyl isothiocyanate, NAMIT, Fig. 1) were therefore evaluated for the HPLC separation of the diastereomeric forms of labetalol. As seen in Fig. 3 and Table I, both reagents gave excellent separation of the diastereomers of labetalol, and once again the derivatives gave good band shapes. Excess reagent eluted before the derivatives and did not interfere with the analysis (Fig. 3). It was observed that for all three reagents (PHEDIT, BIT, and NAMIT) the *R,S/S,R* isomers gave derivatives that eluted before the derivatives of the *R,R/S,S* isomers (Figs. 2 and 3). BIT and NAMIT are readily available and inexpensive, and may therefore have utility in diastereomer separations by HPLC. It is also noteworthy in this context that the only other reported HPLC separation of the racemates of labetalol, while involving no derivatization, achieved less-than-complete resolution [9].

In conclusion, isothiocyanate reagents are useful in HPLC separations of the stereoisomers of labetalol. Studies on the applicability of these procedures to the analysis of the isomers in biological media are in progress.

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