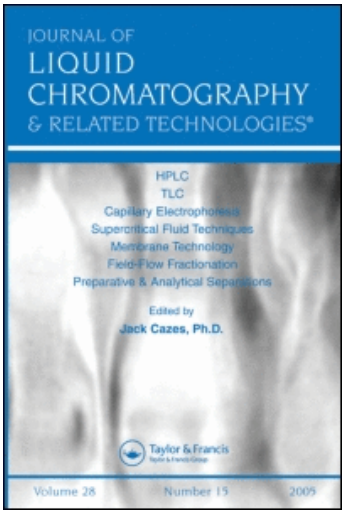

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Determination of the Enantiomeric Composition of a Novel Topically Active Carbonic Anhydrase Inhibitor by HPLC

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DETERMINATION OF THE ENANTIOMERIC COMPOSITION OF A NOVEL TOPICALLY ACTIVE CARBONIC ANHYDRASE INHIBITOR BY HPLC

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ABSTRACT

An HPLC method for the determination of the SS/RR stereoisomeric content of dorzolamide drug substance is described. The method employs precolumn derivatization with the chiral reagent (S)-phenethylisocyanate resulting in diastereomeric derivatives. The derivatives are separated under normal phase conditions employing a silica column. Low level detection (0.1%) of the minor RR enantiomer is achieved.

INTRODUCTION

Elevated intraocular pressure in patients with ocular hypertension or open angle glaucoma can be alleviated by inhibition of carbonic anhydrase II isozyme (CAII), thereby modulating the rate of aqueous humor formation.[1,2] Therapeutic administration of oral carbonic anhydrase inhibitors (CAI's), while extremely effective, can result in serious systemic side effects which may diminish patient compliance or prevent their use entirely.[3,4]

Recently, topically effective CAI's have generated much interest due to potential alleviation of adverse experiences and inherent target organ specificity.[5] Certain novel derivatives of thienothiopyran-2-sulfonamide were chosen for evaluation as CAI drug candidates, with emergence of dorzolamide as the lead therapeutic entity.[6-9] Animal models have demonstrated that greater inhibition resides in the antipode of dorzolamide having the (S) configuration at the 4-ethylamino position and x-ray crystallographic studies of the inhibitor-enzyme complex show a more favorable orientation of the 4-(S) stereoisomer within the enzyme compared to the 4-(R).[6,7,10]

Support of process development aimed at the enantioselective synthesis of dorzolamide as the 4S,6S antipode required a rapid assay for the determination of the 4S,6S and 4R,6R enantiomeric pair in the bulk drug substance. Several chromatographic investigations in support of pharmacological studies have been reported, culminating in the successful separation of the potential stereoisomers of dorzolamide by precolumn formation of the diastereomeric methylnapthyl urea derivatives.[11,12] Acceptable resolution could only be achieved by employing a Pirkle Π-acceptor type chiral column. This work describes the investigation of a chiral benzyl isocyanate as a precolumn derivatizing reagent with subsequent separation of the diastereomeric derivatives of the 4S,6S//4R,6R stereoisomeric pair within an achiral HPLC chromatographic environment.

MATERIALS

Apparatus

Chromatographic studies were performed using a Hewlett-Packard model 1090 HPLC equipped with a photodiode array detector. Mass spectra were acquired with a Finnigan model 710 SSQ using a thermospray interface. ^{1}H NMR spectra were recorded using a Brucker model 250 FTNMR. Elemental analyses were acquired from the microanalysis section of the Merck Research Laboratories.

Chemicals

Ammonium hydroxide, sodium bicarbonate, and acetic acid were reagent grade from Fisher Scientific (Bridgewater, N.J.). Ethyl acetate, methylene chloride, methyl tert-buty ether, acetonitrile, and heptane were from Burdick

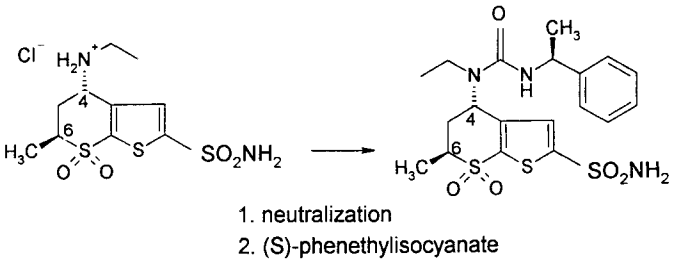

Figure 1. Derivatization of dorzolamide with (S)-phenethylisocyanate.

and Jackson (Muskegon, Wi.). S-(-)-phenethylisocyanate was prepared by the method of Cairns[13] using purified (R)-phenethylamine as the starting material. Samples of the stereoisomers of dorzolamide were supplied by the process research department of the Merck Research Laboratories.

METHODS

The derivatization scheme is shown in Figure 1. To 20 mg of dorzolamide was added 2 mL of a saturated sodium bicarbonate solution. The bicarbonate solution was extracted twice with 2 mL ethyl acetate. The ethyl acetate extracts were combined and evaporated to dryness with a stream of nitrogen. The residue was dissolved in 2 mL acetonitrile and 2 drops of (S)-phenethylisocyanate was added. The solution was evaporated to dryness, and the residue was reconstituted in 10 mL of a 9:1 ratio of 3% acetonitrile in methyl tert-butyl ether:acetic acid. The solution was chromatographed using a Zorbax Sil column (Mac-Mod, Chadds Ford, Pa.) 150 mm x 4.6 mm, 5 micron nominal particle size, with a mobile phase of 65% of 3% acetonitrile in methyl tert-butyl ether and 35% heptane at a flow rate of 2 mL/min. The detection wavelength was 254 nm. The injection volume was 10 μL.

Preparation of the SSS Diastereomer

250 mg of dorzolamide was neutralized with 5 mL of a solution of 10% ammonium hydroxide and extracted twice with 5 mL ethyl acetate. The ethyl acetate extracts were dried over anhydrous sodium sulfate and filtered. The filtrate was evaporated to dryness and dissolved in 3 mL acetonitrile. 300 mg of (S)-phenethylisocyanate was added and the reaction was allowed to proceed for 0.5 hour at room temperature. The mixture was evaporated and

triturated with methyl tert-butyl ether/petroleum ether. ^{1}H NMR (250 mHZ, DMSO-d_6, ppm) 1.08 (t, 3H), 1.38 (m, 6H), 2.37 (m, 1H), 2.72 (m, 1H), 3.05 (m, 1H), 3.28 (m, 1H), 3.90 (m, 1H), 4.90 (m, 1H), 5.37 (m, 1H), 6.84 (d, 1H), 7.19 (s, 1H), 7.22-7.31 (m, 5H), 8.06 (s, 2H). MS (flow injection, carrier 50% aqueous acetonitrile containing 0.2% trifluoracetic acid, 1.2 mL/min) m/e 472 $(M+H)^+$, 100%, m/e 393, 40%, m/e 325, 90%. Melting point 161.5-163°C. Analysis. Calculated for $C_{19}H_{25}N_3S_3O_5$: C, 48.38; H, 5.34; N, 8.91; S, 20.40. Found: C, 48.36; H, 5.30; N, 8.83; S, 20.27.

Preparation of the RRS Diastereomer

The RRS diastereomer was prepared in the same fashion as the SSS derivative. The derivative spontaneously crystallized from the reaction mixture. It was collected on a sintered glass funnel, washed with cold methyl tert-butyl ether, and sucked dry. ^{1}H NMR (250 mHZ, DMSO-d_6, ppm) 1.10 (t, 3H), 1.39 (m, 6H), 2.37 (m, 1H), 2.72 (m, 1H), 3.15 (m, 1H), 3.30 (m, 1H), 3.90 (m, 1H), 4.88 (m, 1H), 5.30 (m, 1H), 6.82 (d, 1H), 7.19 (s, 1H), 7.22-7.35 (m, 5H), 8.06 (s, 2H). MS m/e 472 $(M+H)^+$, 100%; m/e 393, 60%; m/e 325, 80%. Melting point 241-242°C. Analysis. Found: C, 48.18; H, 5.18; N, 9.12; S, 20.34.

Purification of (R)-Phenethylamine

0.2 moles of (2S,3S)-(-)-tartaric acid was added to 400 mL methanol and the mixture heated. To the hot solution was added 0.2 moles R-(+)-phenethylamine. The solution was cooled and allowed to crystallize by standing at room temperature overnight. The crystallized salt was collected on a sintered glass funnel, washed with cold methanol and sucked dry. The salt was dissolved in 100 mL water and 12.5 mL of a 50% solution of sodium hydroxide was added to liberate the amine. The amine was extracted with 75 mL ethyl ether and the extract dried over anhydrous magnesium sulfate. The dried solution was filtered and the R-(+)-amine collected by distillation. Boiling point 186-187°C. $[\alpha]^{20}_D = +38°$.

Enantiomeric Purity of (S)-Phenethylisocyanate

The enantiomeric purity of (S)-phenethylisocyanate was ascertained by derivatization of methylene chloride solutions of a racemate and the pure (S)-stereoisomer of timolol. The derivatives were chromatographed using a Zorbax

Sil column, 150 mm x 4.6 mm, with a mobile phase of 9:1 methylene chloride:acetic acid. operated at a flow rate of 1.2 mL/min with UV detection at 254 nm. A sample load of 30 μg was used.

RESULTS AND DISCUSSION

The use of phenethylisocyanate as a chiral derivatizing reagent has been demonstrated for the stereochemical determination of a number of chiral primary and secondary amines, such as alkaloids,[14,15] β-adrenoceptor antagonists,[16,17] amphetamines,[18] and dealkylated phenothiazines,[19] employing spectroscopic or chromatographic measurement. Some distinct advantages include reaction under mild conditions affording high yield and lack of racemization. In the case of β-adrenoceptor antagonists, exclusive reaction with hindered secondary amines at the expense of unhindered primary hydroxyls, even with excess derivatizing reagent, was noted.

In order to alleviate inaccurate assessment of the RR levels in dorzolamide due to contamination of the derivatizing reagent with the undesired antipode, high enantiomeric purity of the (S)-phenethylisocyanate was achieved by treatment of the precursor (+)-phenethylamine with (+,+)-tartaric acid. The purity of the isocyanate was checked by derivatization with a β-adrenergic blocking agent, timolol, having high enantiomeric purity.

After confirmation of the retention time of the (R)-timolol-(S)-phenethylisocyanate derivative, none of the (R)-phenethylisocyanate, present as the (S)-timolol-(R)-phenethylisocyanate derivative was observed (detection limit <0.05% by area).

The structure of both the SSS and RRS derivatives of dorzolamide was confirmed by spectral and elemental analysis. Vast differences in solubility were noted. The RRS derivative precipitated out of the reaction medium while the SSS derivative was completely soluble and required trituration for isolation and identification. The difference in the observed melting point of the diastereomers was nearly 80°C.

Under mild ionization afforded by thermospray MS, some fragmentation was noted. The protonated molecular ion, m/e 472, was observed as the base peak. The fragment at m/e 393 was attributed to loss of the sulfonamide side chain $(M\text{-}SO_2NH_2 + 2H)^+$ and the fragment at m/e 325 was assigned to the loss of the methylbenzylamido side chain $(M\text{-}CONHCH(CH_3)Ph + 2H)^+$.

Kinetic Study

The kinetics of the reaction of dorzolamide with (S)-phenethylisocyanate were studied at 15, 30, and 45°C. A four fold molar excess of the derivatizing reagent was used. The reaction was found to be complete within ten minutes at all three temperatures. The reaction kinetics of some secondary amine phenothiazines have been reported under similar reaction conditions with reaction completion noted within 0.5 hour.[19]

Chromatography

The separation of the SSS/RRS derivatives was initially attempted under reversed phase conditions using a C_8 or C_{18} column. However, no appreciable selectivity was noted. The separation of the 1-napthylethylisocyanate derivative of dorzolamide under reversed phase conditions has resulted in only partial separation of the SSS/RRS diastereomeric pair at a k' of 30 for the first eluted derivative.[11] Conversely, we observed better separation results under normal phase conditions, consistent with previous studies.[12]

The best separation was accomplished with a ternary mobile phase of methyl tert-butyl ether, heptane, and acetonitrile. A selectivity factor of 1.9 was achieved at a k' for the first eluted derivative, the SSS diastereomer, of 2.0. A separation of the derivatized SS/RR racemate is shown in Figure 2. Although it is generally preferable to have the minor component of a mixture as the species eluting first for enhanced detectability,[20] the separation was good enough to allow detection of the derivatized RR enantiomer at a k' slightly greater than the major SS derivative.

Possible interferences from the derivatization chemistry were investigated by individual workup of the derivatizing reagent only and the underivatized racemate or pure 4S,6S enantiomer only. No chromatographic interferences were noted.

A mixture containing all potential stereoisomers of dorzolamide was derivatized and chromatographed. Although 4 bands were expected, 3 were observed.

Subsequent studies have shown that the 4R,6S species co-elutes with the 4R,6R stereoisomer.[21] However, the 4R,6S isomer can be separated under reversed phase conditions, and the level of the 4R,6R isomer determined by simple subtraction.

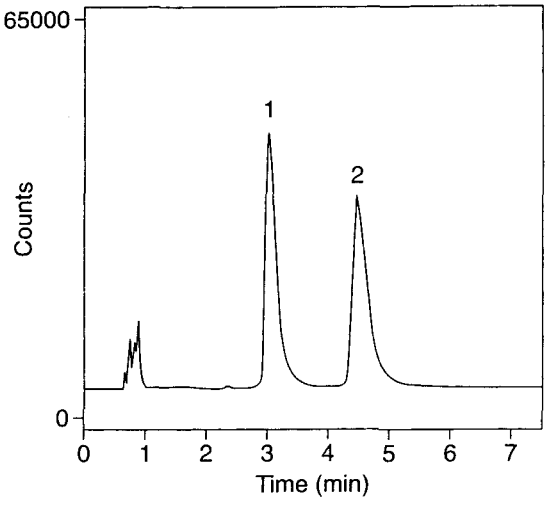

Figure 2. HPLC chromatogram of a 4S,6S//4R,6R racemate of dorzolamide after derivatization with (S)-phenethylisocyanate. Peak 1 is the SSS diastereomer and peak 2 is the RRS diastereomer. The separation was performed at 25 °C.

Influence of Temperature

The effect of temperature upon retention in reversed phase HPLC employing alkyl stationary phases such as octyl or octadecyl has been investigated with retention mechanisms usually giving rise to well behaved free energy relationships as evidenced by linear Van't Hoff plots.[22] Alternatively, retention behavior in normal phase HPLC has been shown to be variable under the influence of temperature, particularly in instances where a non-polar carrier, such as an n-alkane, is doped with a small amount of a polar modifier. On a silica sorbent, the retention of C_{18} and C_{22} fatty acids has been observed to either increase or decrease as a function of temperature and mobile phase modifier, and for an aromatic diester retention has been shown to either increase, decrease or be invarient as solvent and temperature were varied.[23] The retention of a rigid spirolactam on a chiral sorbent exhibited both an increase and decrease in k' at constant mobile phase composition over the temperature range of the study.[24] Since the composition of the mobile phase in this study contains a small amount of the polar modifier acetonitrile, the effect of temperature upon retention was investigated in the range from 0 to 70°C.

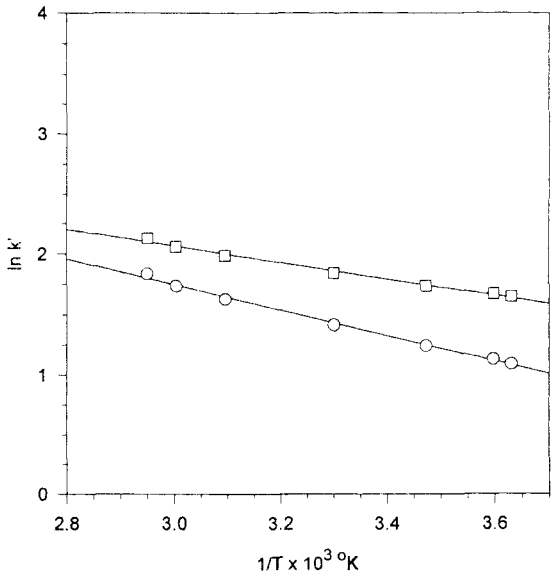

Figure 3. Van't Hoff relationship for the SSS (O) and RRS (□) derivatives of dorzolamide using a mobile phase of 1.8% acetonitrile, 63.2% methyl tert-butyl ether and 35% heptane.

The Van't Hoff relationship illustrating the dependance of retention upon temperature is shown in Figure 3. Linear behavior was noted in the temperature range studied for both derivatives. Surprisingly, retention was observed to increase as the temperature of the separation was increased, giving rise to a positive enthalpy term for the separation. The calculated enthalpy was 2.04 Kcal/mole°K for the SSS diastereomer and 1.33 Kcal/mole°K for the RRS diastereomer.

An increase in retention as column temperature increases in normal phase HPLC has been ascribed to the intimate adsorption behavior of the polar modifier. At higher temperatures, adsorbed polar modifier may be preferentially desorbed from the support resulting in increased exposure of active binding sites.[23-26] Although the ε_0 of acetonitrile is not as great as ethanol, propanol, or water, polar components of predominently non-polar mobile phases exhibiting this behavior, it is polar enough to undergo desorption from the silica surface at higher temperatures resulting in stronger adsorption of both diastereomers onto the support. The increased enthalpy of adsorption of the SSS diastereomer relative to the RRS counterpart may be due to the energy

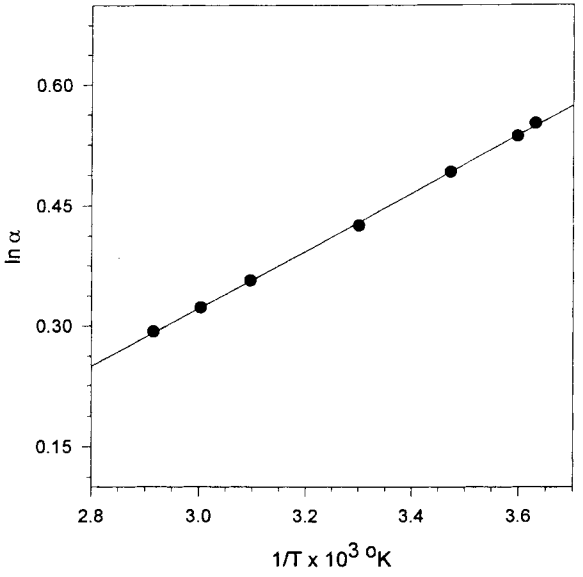

Figure 4. Plot of ln ∝ versus 1/T according to eq. 1 for the SSS and RRS diastereomeric derivatives of dorzolamide.

requirement of mobile phase desolvation to stationary phase adsorption. In the synthetic preparation of both compounds, the SSS diastereomer was readily soluble in a variety of solvents, in direct contrast to the RRS diastereomer. The solvation behavior may also explain the observed retention order. The RRS diastereomer, having a greater solvation requirement, may prefer the silica sorbent rather than a solvated mobile phase environment.

The relationship:

$$\Delta\Delta G = -RT \ln \alpha \qquad (1)$$

where G is the free energy and α is the chromatographic selectivity factor, has been shown to be a diagnostic term for the mechanism of retention.[24] Variability in mechanism, such as a stationary phase conformational change, usually is indicated by non-linear behavior. The relationship found in this study is shown in Figure 4. Linear behavior was noted over the temperature range studied, indicating that the chromatographic process, even with varying amounts of adsorbed polar modifier, is constant and influences both components in the same manner.

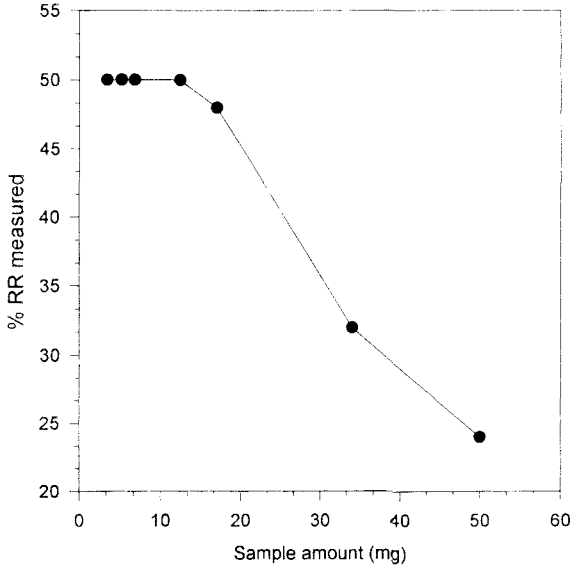

Figure 5. The effect of sample size on the measurement of the 4R,6R stereoisomer of dorzolamide. The sample derivatized was racemic 4S,6S//4R,6R.

Effect of Sample Size

Due to vast solubility differences noted between the SSS and SSR diastereomers, the effect of sample size upon the measurement accuracy was investigated. A sample of racemic 4S,6S/4R,6R was used. Various sample sizes were derivatized and the enantiomeric composition of the sample was measured. The results of the study are presented in Figure 5.

The expected stereochemical composition of the mixture was obtained up to a sample size of 10 mg. At sample amounts greater than 10 mg, the measured level of the 4R,6R stereoisomer decreased due to precipitation of the RRS derivative in the reconstituted sample. When 18 mg of sample was derivatized, slight cloudiness of the reconstituted solution was observed, while at higher amounts of 34 and 50 mg, heavy precipitation was noted. Analysis of the precipitate by HPLC indicated concomitant enrichment in levels of the RRS diastereomer. The study indicated that samples containing greater than 5 mg of the 4R,6R stereoisomer may give rise to inaccurate results.

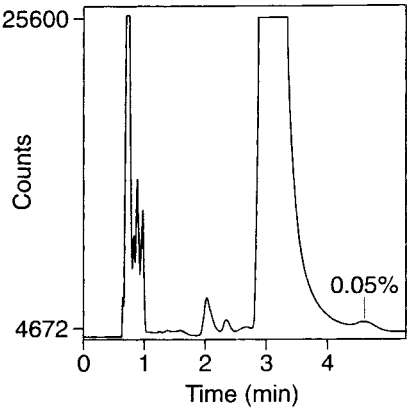

Figure 6. Low level (0.05%) HPLC measurement of the 4R,6R stereoisomeric content in dorzolamide drug substance.

It is interesting to note that an attempt to enhance detection of the minor enantiomer by derivatization with (R)-phenethylisocyanate to invert the retention order was not successful due to the insolubility of the SSR diastereomer in the sample diluent.

Analytical Figures of Merit, Precision, and Repeatability

The response of the SSS diastereomer was investigated in the range from 6.5 ng to 30 μg injected on-column. Linear behavior was observed in this range. The response was given by $Y=3.71\bullet10^5$ X - 7828 ($r^2=0.99998$). The limit of detection for the RRS derivative was extrapolated from the detection limit of the SSS derivative which was 1.3 ng at a signal to noise ratio of 3. The limit of quantitation of the derivatized 4R,6R stereoisomer was 0.02% based upon a 20 μg sample load of the derivatized 4S,6S stereoisomer. A separation showing detection of the RR stereoisomer at the 0.05% level is shown in Figure 6.

The precision of the assay was investigated by repetitive injections of a sample containing 0.3% of the 4R,6R stereoisomer. For 5 injections, the % of the 4R,6R stereoisomer was unchanged. Day to day repeatability was investigated by derivatization of 3 lots of dorzolamide over a 3 day period. In all cases, the % of the 4R,6R stereoisomer was unchanged.

CONCLUSIONS

(S)-phenethylisocyanate, of high enantiomeric purity, was found to react rapidly with the secondary amine of the SS/RR enantiomers of dorzolamide resulting in the formation of diastereomeric ureas allowing for the measurement by HPLC of the RR stereoisomer at low levels. Vastly different properties of the diastereomeric derivatives, particularly the low solubility of the RRS compound, can influence the accuracy of the assay for samples containing appreciable amounts of the RR enantiomer due to precipitation of this derivative in the sample diluent. Under normal phase conditions employing a low concentration of the polar modifier acetonitrile, retention for both diastereomers was found to increase as the temperature of the separation was increased. This was attributed to desorption of acetonitrile from the adsorbent, resulting in the exposure of active binding sites on the silica.

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