Pharmaceutical Research, Vol. 11, No. 3, 1994

Analytical Chiral Separation of the Stereoisomers of a Novel Carbonic Anhydrase Inhibitor and Its Deethylated Metabolite, and the Assignment of Absolute Configuration of the Human Metabolite and Chiral Degradation Products[1]

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Received May 6, 1993; accepted September 21, 1993

Several approaches to the separation of four stereoisomers, **1–4**, of a novel, topically active, carbonic anhydrase inhibitor, **1**, with two chiral centers in the molecule and four isomers, **5–8**, of its chiral metabolite, **5**, were evaluated. These methods include nonchiral derivatization followed by separation on chiral stationary phases (CSPs) and chiral derivatization and separation on nonchiral columns and on CSPs. Baseline separation of stereoisomers **1–4** was achieved in less than 15 min after chiral derivatization with (*S*)-(+)-1-(1-naphthyl)ethyl isocyanate (NEIC) and chiral chromatography on a (*R*)-*N*-(3,5-dinitrobenzoyl)phenyl glycine (DNBPG) column under normal phase (NP) conditions. Similarly, isomers **5–8** were baseline separated in less than 20 min after derivatization with NEIC and chromatography on nonchiral (nitrophenyl) and chiral [(*S*)-(3,5-dinitrobenzoyl)leucine; DNBL] columns in series under the same NP chromatographic conditions. Only partial separation of the diastereomeric derivatives was observed on a variety of nonchiral columns. In addition, all other direct and indirect chiral separation approaches gave only partial separation of at least two stereoisomers within the group of **1–4** or **5–8**. The details of chiral separations using various methods and separation (α) and capacity factors (k') of the derivatized isomers **1–8** on a series of chiral and nonchiral columns are presented. Using these methods, the absolute configuration of the human metabolite of **1** was established as S_1S_2 (**5**), and the heat (HD) and light (LD) degradation products of **1** as R_1S_2 (**3**) and S_1S_2 (**5**), respectively.

KEY WORDS: chiral derivatization; chiral separation; high-performance liquid chromatography; diastereomers; absolute configuration; metabolite; chiral degradation products; chirality.

INTRODUCTION

Compound **1** [(−)-(*SS*)-4-ethylamino-5,6-dihydro-6-methyl-7,7-dioxide-4H-thieno(2,3-b)thiopyran-2-sulfonamide (Fig. 1)] belongs to a class of recently discovered topically active carbonic anhydrase inhibitors (1,2). It is being evaluated in our laboratories as an antiglaucoma agent. The molecule of **1** contains two chiral centers and can exist in four stereoisomeric forms, **1–4**. All preclinical and clinical studies were performed using the single stereoisomer **1** with absolute configuration S_1S_2 around the two chiral centers of the molecule. To study the potential for *in vivo* inversion of configuration at one or both chiral centers of **1**, a method for the chiral separation of **1–4** based on chiral derivatization with (*S*)-(+)-1-(1-naphthyl)ethyl isocyanate (NEIC) and reverse-phase (RP) chromatography had been developed previously (3). However, this method had several limitations including only partial separation of isomers **1** and **2**, a long analysis time (90 min) leading to relatively broad peaks, and a poor detection sensitivity. In addition, separation of isomers **5–8** was unsuccessful under RP chromatographic conditions. Therefore, to establish the absolute configuration of the human metabolite of **1**, and two degradation products corresponding to one of the isomers of parent compound (**2–4**) and to one of the isomers of the deethylated analogue (**5–8**), development of a new methodology capable of baseline separation of all isomers **1–8** was necessary.

Several direct and indirect approaches to the separation of stereoisomers **1–8** were evaluated and included

- separation on chiral stationary phases (CSPs) of nonchirally derivatized isomers and
- indirect separation after derivatization with a chiral reagent and separation of diastereomers using nonchiral normal phase (NP) chromatography or chiral chromatography in the NP mode.

The direct chiral separation mode is usually preferred (4) and is used extensively for separation of stereoisomers in a simple sample matrix. Its application to multisample analyses of biological samples in support of clinical pharmacokinetic studies seems to be rather limited due to the limitations in the chromatographic conditions utilized, difficulty in resolving endogenous interferences from the compound of interest (5), low efficiency of chiral columns (plate number) and low flow rates resulting in long retention times, and limitations in the composition of the mobile phase (those suitable for NP or low organic contents) which preclude the use of some detector, e.g., the electrochemical detector. Because of these limitations, the indirect methods based on formation of diastereomers by derivatization are more common for the stereoselective assays in biological fluid and offer more flexibility in choosing adequate nonchiral chromatographic conditions for separation of the diastereomeric derivatives.

In principle, after chiral derivatization, the mixture of diastereomers should be separated under nonchiral chromatographic conditions. However, in the case of stereoisomers **1–4**, only partial separation of the diastereomeric urea derivatives derived from **1** and **2** was observed under nonchiral RP conditions (3), and separation of the analogous derivatives **5–8** was not effective. Since the deethylated analogue of **1** was identified both as a human metabolite of **1** and as a degradation product (LD) formed after exposure of an ophthalmic solution containing **1** to ambient light, the assignment of absolute configuration of these products required baseline separation of **5–8**. In addition, a heat degradation (HD) product of the ophthalmic solution of **1** corresponding to one of the stereoisomers **2–4** was also identified, and the availability of a method more effective than RP for

[1] Presented in part at the Third International Symposium on Chiral Discrimination, Tubingen, Germany, October 1992 (Abstract No. 138).

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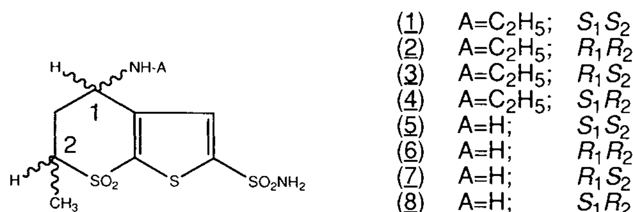

Fig. 1. Chemical structures of the stereoisomers of **1** and its deethylated analogue **5**.

the separation of **1–4** was desirable. This paper describes the development of the NP methods capable of baseline separation of **1–4** and **5–8** in a short analysis time (<25 min) and the assignment of the absolute configuration of the metabolite and both LD and HD products of **1**.

MATERIALS AND METHODS

Materials

Compounds **1–8** were synthesized at the Department of Medicinal Chemistry of Merck Research Laboratories (West Point, PA) by Dr. G. S. Ponticello and his group and were available either as single enantiomers (**1**, **3**, **4**, **5**) or as racemic [(**1** + **2**), (**5** + **6**)] and diastereomeric (**5** + **6** + **7** + **8**) mixtures. The absolute configuration of the isomeric standards was established by single-crystal X-ray analysis (6) and/or chemistry from chiral precursors of known absolute configuration. Nonchiral derivatizing reagents, i.e., benzyl isocyanate (BIC) and 1-naphthyl isocyanate (NIC), and chiral (+)-(*S*)-1-(1-naphthyl)ethyl isocyanate (NEIC) were all purchased from Aldrich (Milwaukee, WI). All other reagents and solvents were of the highest available purity and/or HPLC grade and originated from Fisher Scientific (Fair Lawn, NJ). Several nonchiral and chiral columns were evaluated. The following nonchiral columns were used:

(A) Nucleosil-NO_2 (nitrophenyl, 250 × 4.6 mm, 5 μm, 100 Å) from Keystone Scientific (Bellafonte, PA),
(B) phenylethyl (Bakerbond, 250 × 4.6 mm, 5 μm, 100 Å) from Baker (Phillipsburg, NJ), and
(C) DL-dinitrobenzyl glycine (DL-DNBPG; 250 × 4.6 mm, 5 μm, 100 Å, covalently bonded to silica) from Regis (Morton Grove, IL).

Two chiral Pirkle columns were used:

(D) (*R*)-3,5-dinitrobenzoyl glycine [(*R*)-DNBPG; 250 × 4.6 mm, 5 μm, 110 Å), from Regis and
(E) (*S*)-3,5-dinitrobenzoyl leucine [(*S*)-DNBL; 250 × 4.6 mm, 5 μm, 110 Å), also from Regis.

Samples of ophthalmic solutions, 2% in **1**, were prepared by Dr. P. Quint (Merck-Chibret, France) and exposed to light (LD) or to heat (HD) or left untreated (UT) as standards. The "heat degradation" was performed in a controlled-temperature oven at 50°C for 6 months, whereas "light degradation" had occurred after 4 months of storage at ambient laboratory light in 5.5-mL transparent polyethylene oval ocumeters (3.5-mL fill volume). The LD product was identified by nonchiral chromatography as **5** or its enantiomer **6**, whereas the HD product was identified as an isomer **3** or **4** of **1** (7). These ophthalmic solutions were evaluated to establish the absolute configuration of the LD and HD products.

Instrumentation

The Waters Associates 703 HPLC system equipped with a 730 data module, a 720 system controller, a WISP 710B autosampler, and a 6000A chromatographic pump (Waters-Milipore, Milford, MA) was used for all analyses. An Applied Biosystems (Foster City, CA) 785 absorbance detector was used as a UV detector (λ_{det} = 252 nm). The detector output signal was interfaced either to a Hewlett-Packard Laboratory Automation System (HP 3357 LAS, Palo Alto, CA) or to a PE-Nelson (Cupertino, CA) Access-Chrom data system via a PE-Nelson 900 series interface.

Chromatographic Conditions

Mobile phases with various ratios of hexane, methylene chloride, and methanol were initially evaluated. The best separation on a variety of columns was generally achieved using a 50:45:5 (v/v/v) mixture of these solvents, respectively. Instead of evaluating the effect of changes in the mobile phase composition on the separation of isomeric pairs on any given column, the separation of isomers on various CSPs and nonchiral columns was evaluated in a systematic fashion using the same mobile phase. The mobile phase was filtered through a nylon filter (0.2 μm) prior to use. The flow rate was 2 mL/min. The columns were operated at ambient temperature (approximately 22°C). Injection volumes varied from 10 to 100 μL. The order of elution of the derivatized isomers **1–8** was established by separately injecting the resolved and derivatized standards except for **7** and **8**, for which only a mixture of **5** + **6** + **7** + **8** in addition to **5** and racemic **5** + **6** was available. Since the retention time for the deethylated metabolite and LD product was different from that of either **7** or **8** (see Results), the unavailability of resolved standards **7** and **8** did not preclude the assignment of the absolute configuration of the metabolite and LD product.

Extraction of Isomers 1–8 from Whole Blood and Ophthalmic Solution and Derivatization

Metabolite **5** and its isomers **6–8** were extracted from whole blood using a procedure similar to that described earlier for the extraction of **1** (3). Isomers **1–8** were extracted from the ophthalmic solutions in the following manner: A 10-μL aliquot of the solution was diluted to 300 μL with methanol and extracted with 10 mL of toluene–ethyl acetate–2-propanol (49/50/1, v/v/v) after adjusting the pH to 8 with 2 mL of 0.2 *M* phosphate buffer. The samples were shaken for 20 min at 60 strokes/min and centrifuged, and the upper organic layer (9 mL) was evaporated to dryness in a separate tube under a stream of nitrogen at room temperature. To the residue, 300 μL of the derivatizing solution (10 μL of neat NEIC in 2 mL of dry dichloromethane) was added, and after vortexing, the tubes were allowed to stand at room temperature overnight. The solvent was evaporated to dryness under a stream of nitrogen, the residue was dissolved in mobile phase (1 mL), and 30 μL was injected into the HPLC system. In the case of extracts of whole blood, the mixture after derivatization with NEIC was reconstituted in 300 μL mobile phase, and 100 μL was injected onto the column. After spiking to either whole blood or methanol, the standards **1–8** were extracted and derivatized in a similar

manner. A similar procedure was used during derivatization with nonchiral isocyanates wherein NEIC was replaced with BIC and NIC.

RESULTS AND DISCUSSION

Two major approaches to the separation of stereoisomers **1–8** were evaluated and are described below.

Separation on CSPs of Non-Chirally Derivatized Isomers

The first approach was based on the separation of the isomers on selected CSPs after derivatization of the primary or secondary amino group of **1–8** with nonchiral isocyanates BIC and NIC containing an electron-rich benzyl or naphthyl group, respectively.

Reaction of an isocyanate with an amino group of **1–8** was selected for the introduction of an aromatic group based on the good efficiency of reaction between **1–4** and NEIC observed earlier (3). The purpose of introducing an electron-donating substituent in the derivatized analogues was to increase interactions with the electron-withdrawing (3,5-dinitrobenzoyl) substituents present in CSPs D and E and nonchiral phase A and to facilitate isomer separation.

After derivatization with BIC, separation of **1** from **2** and **3** from **4** on column D was not effective, but two diastereomeric pairs (**1** + **2** and **3** + **4**) were separated from each other with selectivity factor ($\alpha = k'_{3+4}/k'_{1+2}$) 1.122 and capacity factor [$k'_{1+2} = (k_{1+2} - t_0)/t_0$] 2.73 for unresolved **1** + **2** (where t_{1+2}, for example, and t_0 are the retention times for the unresolved pair **1** + **2** and a column void volume, respectively). Under the same conditions, almost baseline separation of the enantiomers **5** and **6** was observed with $\alpha = 1.088$ and $k'_5 = 6.15$. Similarly, isomers **7** and **8** were separated from each other ($\alpha = 1.090$) but coeluted with **5** and **6**.

The replacement of a benzyl group with a naphthyl group in the derivatizing reagent (BIC replaced with NIC) significantly improved the separation of the derivatized isomers **1–4**. For example, separation factors (α) on column D were 1.173 for **1** and **2** and 1.269 for **3** and **4**, with k' of 5.78, 6.78, 6.78, and 8.58 for **1**, **2**, **3**, and (or) **4**, respectively. However, as the k' values indicate, separation of **2** from **3** or **4** was not effective. In addition, in the case of NIC-derivatized isomers **5–8**, separation of **5** from **6** and **7** from **8** was not observed (e.g., $\alpha = 1.004$ for **5** vs **6**), and k' values were 12.04, 12.09, and 13.62 for isomers **5**, **6**, and a mixture of **7** + **8**, respectively.

Similar results were obtained on chiral column E, indicating that full separation of all isomers **1–4** and **5–8** using nonchiral derivatization with BIC and NIC and chiral separation on CSPs D and E was not effective; therefore, a second approach based on chiral derivatization and nonchiral or chiral chromatography was evaluated.

Indirect Separation after Derivatization with a Chiral Reagent Using Nonchiral and Chiral Chromatography Under Normal Phase Chromatographic Conditions

Isomers **1–8** were derivatized with chiral isocyanate (NEIC) to form urea derivatives **9–16** (Fig. 2). The structure of derivative **9** was confirmed earlier (3) by various types of spectroscopy on material synthesized on a 100-mg scale.

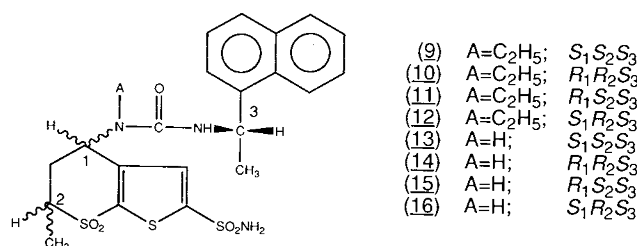

Fig. 2. Chemical structures of the urea derivatives **9–16** formed after derivatization of **1–4** and the deethylated analogues **5–8** with chiral NEIC.

In principle, the diastereomeric analogues **9–16** should be separated under nonchiral chromatographic conditions, and separation under these conditions was initially systematically studied.

Nonchiral Chromatography

Chromatography of **9–16** on three phases (A–C) was investigated to evaluate the effect of the presence of various substituents attached to a nonchiral stationary phase on the separation efficiency. The results of these studies are summarized in the first three entries in Tables I and II. The presence of an electron-withdrawing nitro group in a stationary phase A had a dramatic effect on the separation of both **9–12** and **13–16**, as indicated by comparison of k' and α values for stationary phases A and B. No separation of diastereomers was observed on phase B ($\alpha = 1.00$), probably due to the absence of interaction between the electron-donating (phenyl) group present on stationary phase B and the highly electron-donating characteristics of derivatives **9–16** dominated by the presence of the naphthyl group. The replacement of the phenyl group (phase B) with a nitrophenyl group (phase A) allowed separation of the majority of diastereomers, with α values ranging from 0.88 to 1.07 for **9–12** (Table I) and 0.91 to 1.24 for **13–16** (Table II). Under our chromatographic conditions, baseline separation was observed when α values were less than 0.90 and higher than 1.10. An increase in both k' and α values was observed when a stationary phase with one electron-withdrawing nitro substituent (phase A) was replaced with a stationary phase containing two nitro groups (phase C). However, a complete and

Table I. The Separation (α) and Capacity (k') Factors for Separation of Diastereomers **9–12** Under Nonchiral and Chiral Chromatographic Conditions: Mobile Phase, Hexane/Methylene Chloride/Methanol (50:45:5, v/v/v); Flow Rate, 2 mL/min

	k'				α		
Column	9	10	11	12	9/10	9/11	12/9
A	3.08	2.87	3.40	3.06	1.07	0.88	1.02
B	—[a]	—	—	—	1.00	1.00	1.00
C	4.64	3.96	4.99	4.50	1.17	0.93	0.97
D	4.97	3.51	5.80	4.14	1.42	0.86	0.83
E	4.94	3.09	4.96	3.47	1.60	1.00	0.70
A + D	4.19	3.34	4.83	3.81	1.23	0.87	0.91
A + E	4.38	3.30	4.60	3.64	1.33	0.95	0.83

[a] No retention.

baseline separation of all isomers 1–4 and 5–8 was not achieved when nonchiral stationary phases A, B, and/or C were used. Therefore, separation of derivatives 9–16 was attempted on chiral columns and also on a combination of achiral and chiral columns in series.

Chiral Chromatography

Isomers 1–4, in the form of derivatives 9–12, were baseline separated in less than 15 min on chiral column D (Table I and Fig. 4A). The additional degree of chirality present in D during interaction of diastereomers 9–12 with CSP facilitated separation of isomers 9–12. The absence of this interaction on the otherwise identical, but nonchiral column C prevented baseline separation of all isomers (Table I; e.g., $\alpha = 0.97$ for separation of 12 and 9). On the other hand, separation of the metabolite isomers 5–8 on column D, in the form of derivatives 13–16, was only partially successful, and no separation of 14 and 15 (or 16) was observed.

The efficiency of separation of diastereomers 9–12 was highly dependent on small structural changes of the CSP, as illustrated by the lack of separation of isomers 9 and 11 when DNB-glycine phase D was replaced with DNB-leucine phase E (Table I). On the other hand, separation of the metabolite isomers 13–16 was more effective on phase E in comparison with D (Table II).

To improve even further the separation of metabolite isomers 14 from 15 and 16 from 13, separation was attempted on a combination of chiral and nonchiral columns in series. Inspection of Table II and Figs. 3A and 5A indicates that baseline separation of 13–16 was achieved when columns A and E were used (A + E) in series. The delicate balance required for an effective separation of all eight stereoisomers 9–16 was exhibited by poorer separation of isomers 9–12 under the conditions required for the baseline separation of metabolite isomers 13–16. In the latter case the use of columns A + E was required. Baseline separation of isomers 9 and 11 was not observed ($\alpha = 0.95$; Table I) under these conditions.

Based on all these results, in all subsequent studies, isomers 9–12 were separated using a single chiral column D, whereas separation of 13–16 was performed using a combination of nonchiral and chiral phases A + E.

Table II. The Capacity (k') and Separation (α) Factors for Diastereomers 13–16: Mobile Phase, Hexane/Methylene Chloride/Methanol (50:45:5, v/v/v); Flow Rate, 2 mL/min

Column	k'				α		
	13	14	15[a]	16[a]	13/14	13/15	16/13
A	6.36	5.73	5.13	5.77	1.11	1.24	0.91
B	—[b]	—	—	—	1.00	1.00	1.00
C	7.27	5.30	5.54	7.27	1.37	1.31	1.00
D	12.58	6.37	6.37	11.34	1.97	1.97	0.90
E	11.68	7.45	6.85	10.56	1.57	1.71	0.90
A + D	10.70	7.09	6.75	9.70	1.51	1.59	0.91
A + E	10.03	7.33	6.63	8.90	1.37	1.51	0.89

[a] The order of elution of 15 and 16 is unknown, at present, due to the unavailability of resolved standards.
[b] No retention.

Assignment of the Absolute Configuration of the Deethylated Metabolite of 1

Pre- and postdose whole-blood samples from human subjects participating in the multiple-oral dose pharmacokinetic study were extracted, derivatized with NEIC, and analyzed for the presence of deethylated metabolite using a combination of columns A + E. These analyses revealed that a major single peak was present in the region of metabolite elution and corresponded to diastereomer 13 (Fig. 3).

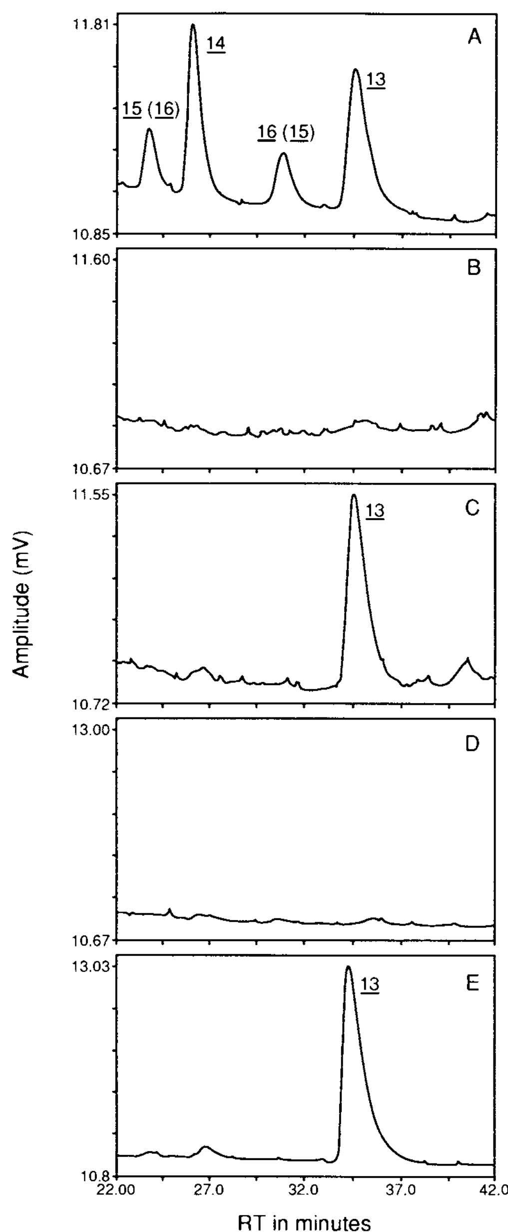

Fig. 3. Separation of four stereoisomers of the deethylated metabolite of 1 after chiral derivatization with NEIC on nitrophenyl/DNBL columns A + E in series. Mobile phase, hexane/methylene chloride/methanol, 50:45:5 (v/v/v). (A) Mixture of derivatized standards. (B, D) Predose whole blood of subjects 106 and 111 after extraction and derivatization. (C, E) As B and D at the end of week 20 of b.i.d. dosing with a 2-mg oral solution of 1.

This indicated that the absolute stereochemistry of the de-ethylated metabolite of 1 was S_1S_2 (5).

Assignment of the Absolute Configuration of the "Heat Degradate" and "Light Degradate" Products Formed in the Ophthalmic Solution of 1

The light-degraded (LD) and heat-degraded (HD) 2% ophthalmic solutions of 1 and an untreated ophthalmic solution standard (UT) were extracted, derivatized with NEIC, and analyzed for the presence of stereoisomers 1–4 and 5–8

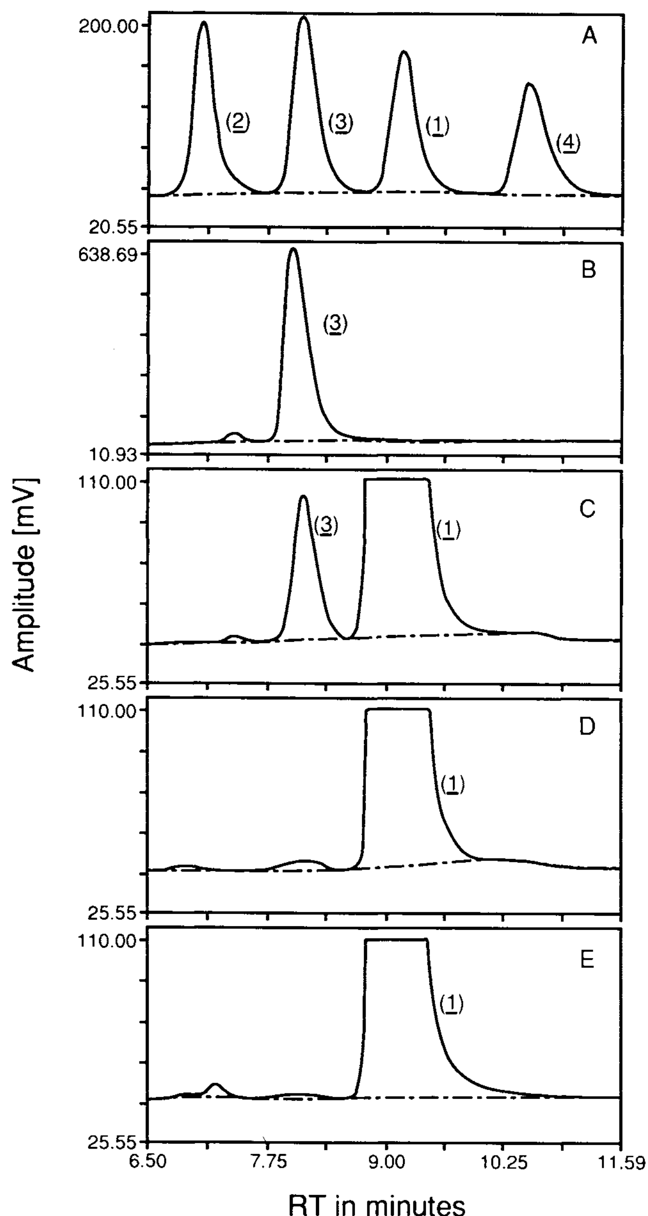

Fig. 4. Assignment of the absolute configuration of the heat degradation product formed in the ophthalmic solution of 1; all isomers were derivatized with chiral NEIC and separated on a chiral DNBPG column, D. Mobile phase, hexane/methylene chloride/methanol, 50:45:5 (v/v/v). (A) Mixture of stereoisomer 1–4 standards. (B) Isomer 3 (R_1S_2) standard. (C) Ophthalmic solution of 1 after "heat degradation." (D) Ophthalmic solution of 1 "untreated." (E) Ophthalmic solution of 1 after "light degradation."

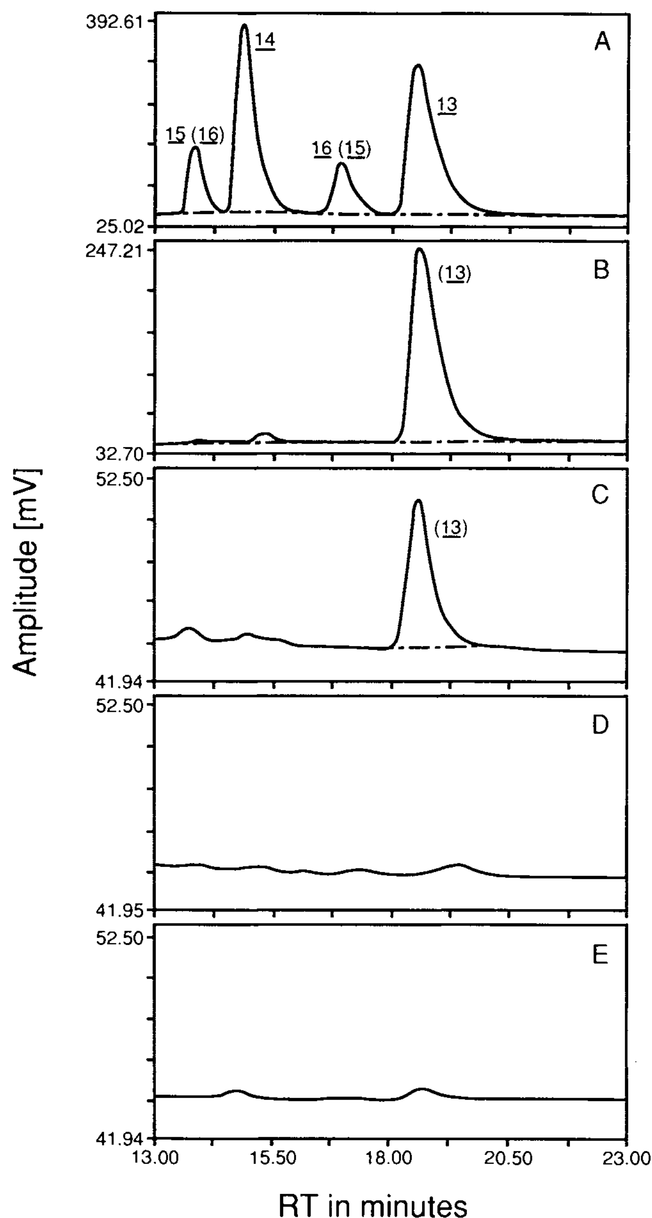

Fig. 5. Assignment of the absolute configuration of the light degradation product of the 2% ophthalmic solution of 1; all isomers were derivatized with chiral NEIC and separated on nitrophenyl/DNBL columns A + E in series. Mobile phase, hexane/methylene chloride/methanol, 43:50:7 (v/v/v). (A) Mixture of stereoisomer 5–8 standards. (B) Isomer 5 (S_1S_2) standard. (C) Ophthalmic solution of 1 after "light degradation." (D) Ophthalmic solution of 1 after "heat degradation." (E) Ophthalmic solution of 1 "untreated."

using chiral column D and columns A + E in series, respectively. Comparison of the retention time of the HD product (Fig. 4C) with the retention times of the individual stereoisomers 1–4 (Figs. 4A and B) clearly indicated that the HD product corresponded to isomer 3, having an R_1S_2 absolute configuration.

The peak area corresponding to this product was 4.88% of the total peak area of the mixture of 1 + 3. The HD product 3 was also present, to a very small extent, in the UT sample (Fig. 4D).

A similar comparison of the retention time of the LD product (Fig. 5C) with the retention times of the individual isomers 5–8 (Figs. 5A and B) indicated that the LD product corresponded to isomer 5, having an S_1S_2 absolute configuration. The absolute configuration of this LD product was not only the same as for the parent compound but also the same as for the human metabolite formed after dosing with 1.

In conclusion, baseline separation of stereoisomers 1–8 required chiral derivatization *and* chiral separation, under normal phase chromatographic conditions, using a single Pirkle column (D) for isomers 1–4 and the combination of a nonchiral nitrophenyl column (A) and a Pirkle column (E) in series for isomers 5–8. The absolute configuration of the deethylated metabolite of 1 present in whole blood of human subjects after oral dosing with 1 was established as S_1S_2 (5). In addition, the absolute configuration of the heat and light degradation products formed in the ophthalmic solution of 1 was established as R_1S_2 (3) and S_1S_2 (5).

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