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High-performance liquid chromatographic determination of the isomeric purity of a series of dioxolane nucleoside analogues[☆]

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ABSTRACT

Racemic (±)-*cis*-2 hydroxymethyl-4-(cytosin-1'-yl)-1,3-dioxolane analogue (BCH-204) exhibited high levels of anti-HIV activity, but also showed cytotoxicity at the active concentration. To examine the possibility of enantiomerically separating the HIV activity from the cytotoxicity in the dioxolane nucleosides, high-performance liquid chromatography using chiral stationary phase columns was examined. The successful separation of dioxolane compounds was demonstrated utilizing optimum conditions of columns, solvents, flow-rates and temperature. In the reversed-phase mode, the cyclodextrin columns Cyclobond I SP and RSP were used to separate the enantiomers of *cis*- and *trans*-(±) dioxolane-C , and the protein column α-AGP was successful in separating enantiomerically *cis*-(±) dioxolane-G and *cis*- and *trans*-enantiomeric forms of (±)-dioxolane-A. In the normal-phase mode, one of the cellulose columns, Chiralcel OJ, successfully separated enantiomerically (±)-dioxolane-T nucleosidic analogue.

INTRODUCTION

The enantiomeric resolution of BCH-189 by high-performance liquid chromatography (HPLC) using the chiral column Cyclobond I Ac [1] produced the promising nucleoside analogue 3TC, which is just completing Phase II clinical trials for the treatment of HIV infection and AIDS. 3TC has an unnatural sugar configuration and was found to be considerably less toxic than the (+)-enantiomer owing to the higher selectivity of the drug for HIV reverse transcriptase over mammalian DNA polymerases [2,3]. Other interesting nucleoside analogues, 1,3-dioxolanes, where the 3'-thia moiety is replaced with oxygen, have been developed by Belleau *et al.* [2]. Both pyrimidine and purine 1,3-dioxolane nucleoside analogues, such as BCH-203, BCH-204 (related racemic diastereoisomers), BCH-344 [4], BCH-571 and BCH-187 (Fig. 1), have shown very interesting anti-HIV properties [5]. Therefore, it was necessary to resolve their enantiomers and study their pharmacological profiles. Further, we have developed a chiral synthesis of these compounds, which requires controlling two epimerizable acetal centres [6]. HPLC was used to evaluate the diastereomeric and enantiomeric purities of key synthetic intermediates and the final products prior to biological evaluation.

HPLC is a useful tool in enantiomeric separations. The availability of many different kinds of

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☆ Dedicated to the memory of the late Professor Bernard Belleau.

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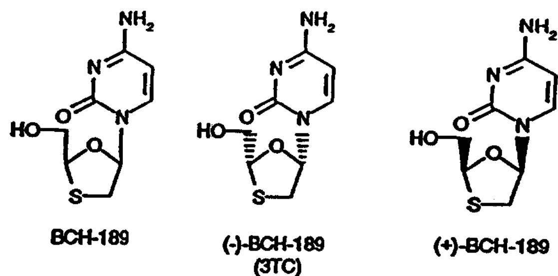

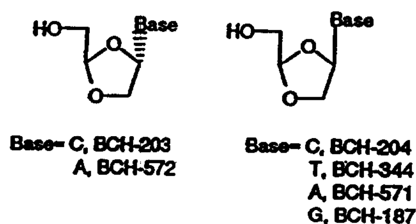

Fig. 1. 1,3-Dioxolane nucleosides with anti-HIV activity.

chiral stationary phases (CSPs) gives chromatographers a wide choice in the selection of CSPs for optimizing enantiomeric separations. In this respect, we have developed procedures using CSPs with suitable mobile phases to monitor the chiral purity of these intermediates and to achieve the resolution of 1,3-dioxolane nucleosides on preparative scale.

EXPERIMENTAL

Materials

The racemic dioxolane compounds, BCH-204, BCH-203, BCH-187, BCH-344, BCH-571 and BCH-572 (Fig. 1) and compounds **1–7** (Fig. 2), were synthesized in our laboratories. The columns Cyclobond I RSP and SP (each 250 × 4.6 mm I.D.) were purchased from Astec (Whippany, NJ, USA), Chiralcel OJ (250 × 4.6 mm)

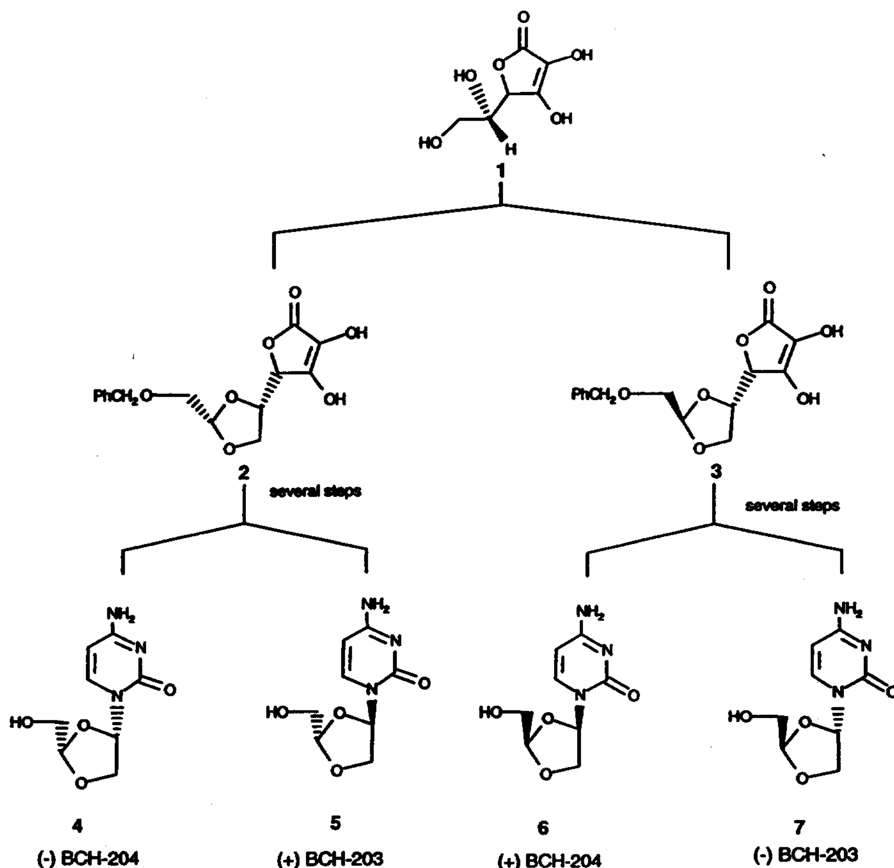

Fig. 2. Synthesis of BCH-203 and BCH-204 stereoisomers from L-ascorbic acid.

from Regis (Morton Grove, IL, USA) and α-AGP from Richard Scientific (Novato, CA, USA). The mobile phase solvents were of HPLC grade from BDH (Montreal, Canada). Triethylamine was purchased from BDH and treated by titrating a 0.05% aqueous solution and adjusting it to pH 7.0 with glacial acetic acid. Buffers were purchased from J.T. Baker (Montreal, Canada) and treated accordingly. Water was of HPLC grade obtained from a Millipore–Waters Milli-Q water-purification system.

Methods

HPLC was performed at various temperatures with a Waters Model 600 multi-solvent delivery system, a WISP 712 automatic injector and a Waters UV detector. The integrator was a Waters Model 740 data module.

RESULTS AND DISCUSSION

One of the important factors in choosing the chiral column and the mode of HPLC for the analysis of a particular compound is the solubility characteristic of that compound [7] . Because BCH-204 is readily soluble in water, reversed phase HPLC was examined. One of the reversed phase chiral columns chosen was in the cyclodextrin series. Cyclobond columns were first evaluated at different concentrations of triethylammonium (TEAA) buffer solutions and various pH values. Separation was achieved with a more derivatized cyclodextrin column than its native column, β-cyclodextrin (Cyclobond I). This derivatized column, called Cyclobond I RSP [(*R*,*S*-hydroxypropyl ether)], has secondary hydroxypropyl residues, which allow further stereospecific hydrogen bonding, enhancing separations of nucleosides [8]. Decreasing the concentration of TEAA provided a separation of enantiomers, but was not optimum. An organic modifier acetonitrile, was chosen to enhance the separation. Acetonitrile has been reported to have a greater affinity than methanol for the cyclodextrin cavity [8]. Fig. 3 demonstrates the HPLC conditions used for the separation of BCH-204.

Further development of this chromatographic

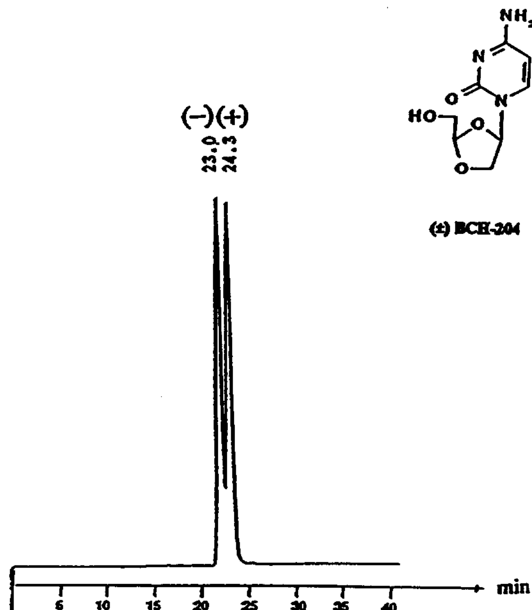

Fig. 3. Resolution of (±)-BCH-204. Column, Cyclobond I RSP (250 × 4.6 mm I.D.); flow-rate, 0.22 ml/min at 0°C; mobile phase, acetonitrile–water (5:95) containing 0.05% of TEAA (pH 7.1) (isocratic); detection, 265 nm; injection, 10 μl of a 1 mg/ml solution.

method was needed when the racemic *trans* form of BCH-204 (BCH-203) had to be separated. Ideally, these two compounds had to be resolved on the same column and under the same conditions in order to calculate the diastereomeric and enantiomeric excess to help to optimize synthetic procedures, as will be demonstrated later. To achieve enantioselectivity for both of these compounds a second Cyclobond I SP column was added in series with the Cyclobond I RSP column. The combination of Cyclobond I SP and Cyclobond I RSP columns was chosen because there was a better resolution for BCH-204 with the Cyclobond I RSP and a better resolution for BCH-203 with the Cyclobond I SP. The concentration of the organic modifier, acetonitrile, was lowered from 5% to 3%, while the other conditions, flow-rate, temperature and buffer concentrations, remained the same. Fig. 2 illustrates why both racemic compounds had to be evaluated under the same conditions.

The chiral synthesis of all the stereoisomers of cytosine dioxolane nucleosides BCH-203 and

BCH-204 was achieved starting from L-ascorbic,(**1**), which provided, in the desired non-selective manner, all the nucleosides in optically pure form for biological testing (Fig. 2). As shown in Fig. 4, all four enantiomers could be resolved in a single chromatogram with all pure stereoisomers of BCH-203 and BCH-204 obtained by the synthetic route superpositioned. However, as there was no baseline separation of the enantiomers of BCH-204 and BCH-203, an accurate determination of the enantiomeric excess or peak purity could not be achieved owing to the tailing of some of the peaks. However, this type of HPLC technique can establish, with further baseline separation, the enantiopurity (% ee) of each of the analogues (**4**, **5**, **6** and **7** in Fig. 2). The chiral synthesis allows the assignment of the absolute configuration of the stereoisomers. Further, generation of optically pure **4** and **5** relied on the efficient separation of diastereoisomers **2** and **3** by fractional crystallization; therefore, an HPLC method was developed to assess the diastereomeric excess of **2** and **3**. An analytical separation of **2** and **3** was achieved on a reverse-phased Whatman Partisil ODS-3 (5 μm) column

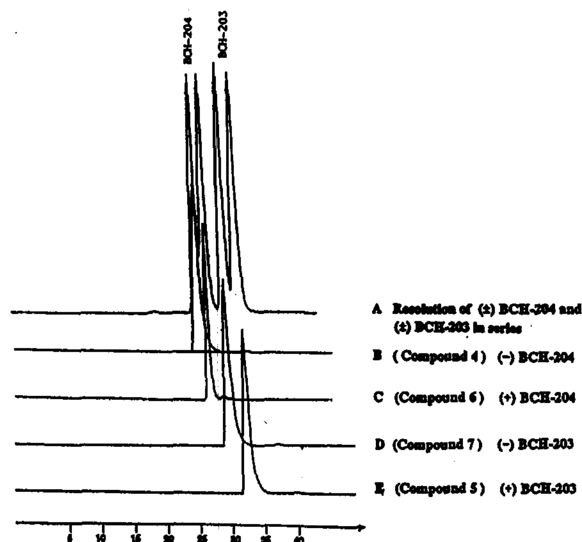

Fig. 4. Resolution of (±)-BCH-204 and (±)-BCH-203 with columns connected in series. Conditions as in Fig. 3, except that two columns, Cyclobond I RSP and Cyclobond I SP columns (each 250 × 4.6 mm I.D.), were placed in series and the mobile phase was acetonitrile–water (3:97) containing 0.05% of TEAA (pH 7.1) (isocratic).

(250 × 4.6 mm I.D.). Fig. 5 shows the chromatogram for (A) the diastereomeric compounds and also the analytical chromatograms after the compounds had been separated preparatively (same column packing but 250 × 22.5 mm I.D.). Compound **2** was obtained in 96.5% purity with a retention time of 31.5 min (Fig. 5B) and **3** in 97.2% purity with a retention time of 33.0 min (Fig. 5C). The high diastereomeric purity of **2** and **3** indicates that this HPLC method is suitable for evaluating the optical purity of dioxolane intermediates early in the synthetic scheme.

Even though the α-AGP (α-acid glycoprotein) protein column did not successfully separate BCH-203 and BCH-204 [(±)-dioxolane-C], other dioxolane compounds, such as the purine analogues, showed optimum peak resolutions. α-AGP consists of a long chain of 181 amino acid residues and 5 carbohydrate units (45% of the total molecule by mass) [9]. This carbohydrate moiety of α-AGP gives the protein a very acidic character [10]. This protein column thus possesses numerous binding sites [11] and is suitable for the enantiomeric resolution of many nucleosides. One such nucleoside, BCH-187 [*cis*-2-hydroxymethyl-4- (guanin-9′-yl) -1,3-dioxolane], achieved excellent separations when a charged modifier, such as 5 m*M* TBAB (tetra-*n*-butylammonium bromide), was added to the sodium phosphate buffer solution of the mobile phase with two α-AGP columns connected in series. TBAB in the mobile phase strongly induces chiral selectivity [12]. Quaternary ammonium compounds such as TBAB increase the retention time and cause competition between the solute and TBAB in binding to the protein column [12]. Initially, in developing the chiral HPLC method for this compound, changing the pH or adding uncharged modifiers such as methanol, 2-propanol or acetonitrile [12] did not succeed in separating these enantiomers, but on the addition of a small amount of TBAB, resolution was achieved as shown in Fig. 6.

Another purine analogue, BCH-571 [*cis*-2-hydroxymethyl-4-(adenin-9′-yl)-1,3-dioxolane], could also be separated on the α-AGP column, but a normal phosphate buffer mobile phase, without the addition of uncharged or charged

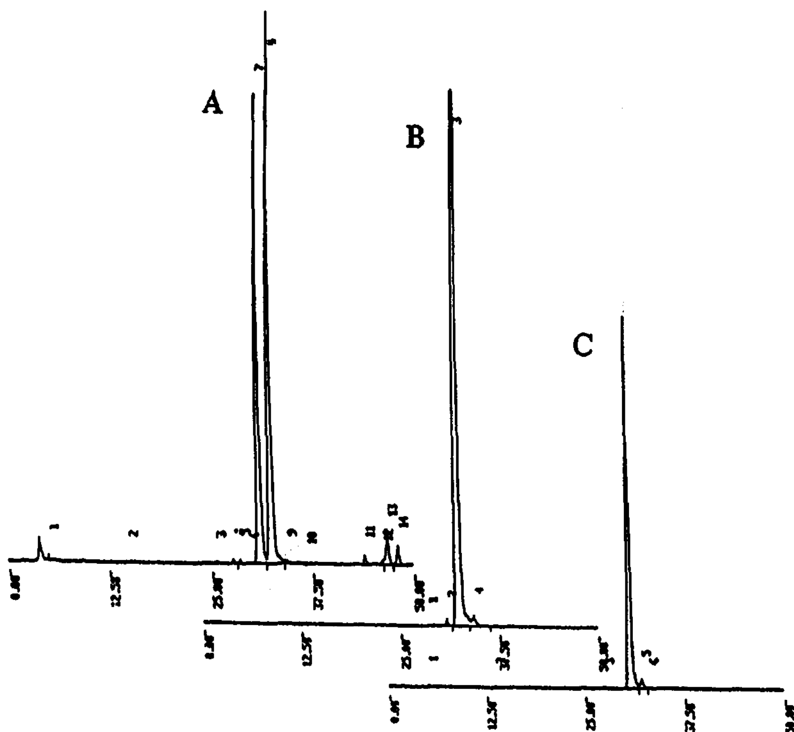

Fig. 5. Chromatograms of (A) diastereoisomers 2 and 3, (B) compound 2 (96.5%) and (C) compound 3 (97.2%). Column, Partisil ODS, 5 μm (250 × 4.6 mm I.D.); flow-rate, 1.0 ml/min; mobile phase, (A) acetonitrile + 0.04%TFA, (B) 0.04% TFA (aqueous), linear gradient from 10 to 50% in 50 min; UV detection at 265 nm.

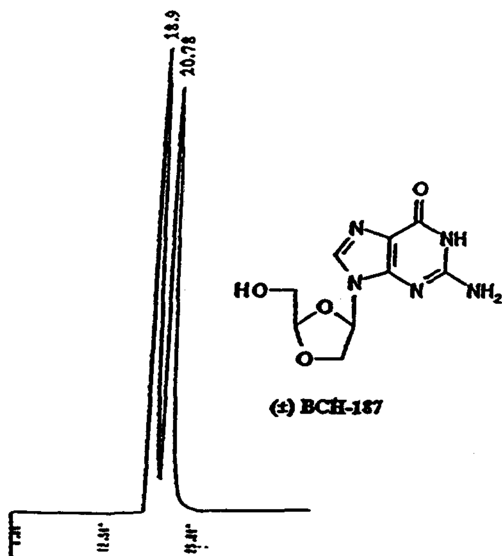

Fig. 6. Separation of (±)-BCH-187. Columns, two chiral α-AGP (100 × 4.0 mm I.D.) in series; temperature, ambient; flow-rate, 0.15 ml/ min; mobile phase, 0.02 *M* NaH_2PO_4–5 m*M* TBAB (pH 7.0) (isocratic); detection, UV at 270 nm.

modifiers, was needed to separate these enantiomers successfully. The results of this separation are shown in Fig. 7. Both of these nucleosides, BCH-187 and BCH-571, were separated at room temperature.

The dioxolane nucleoside BCH-572 [*trans*-2-hydroxymethyl-4- (adenin-9′-yl) -1,3-dioxolane was also resolved on the α-AGP column. The resolution of this compound with a mobile phase of pure phosphate buffer (pH 7.0) did not result in the separation of enantiomers; however, the addition of 0.1 *M* NaCl [12] to the mobile phase and decreasing the temperature slightly to 15°C improved the resolution of BCH-572; the optimum HPLC conditions for this nucleoside are demonstrated in Fig. 8.

For these three purine dioxolane nucleosides we have shown that by adding various uncharged modifiers to the mobile phases and decreasing the temperature, one can optimize enantiomeric

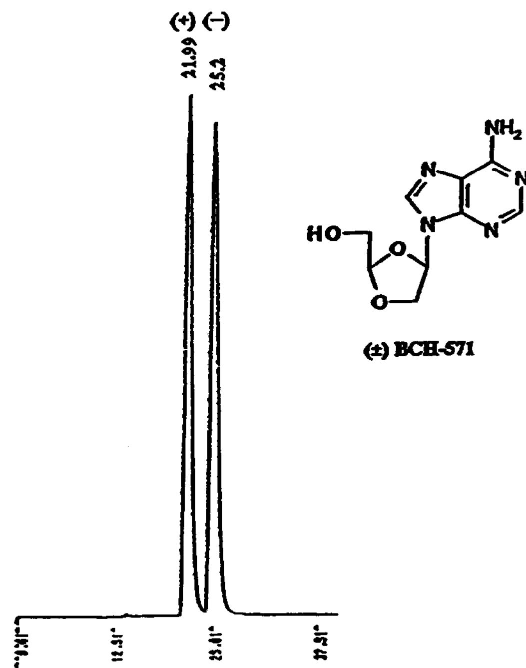

Fig. 7. Enantiomeric resolution of (±)-BCH-571. Conditions as in Fig. 6, but with mobile phase 0.02 *M* NaH_2PO_4 (pH 7.0).

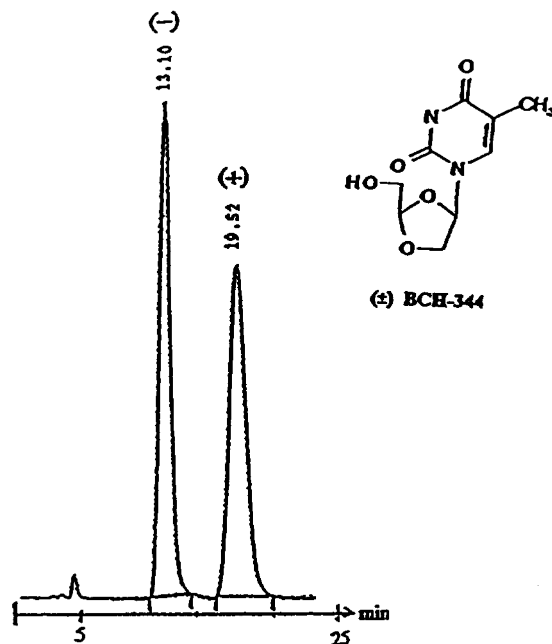

Fig. 9. Chiral separation of (±)-BCH-344.Column, Chiralcel OJ (250 × 4.6 mm I.D.) temperature, ambient; flow-rate, 1 ml/min; mobile phase, 2-propanol–*n*-hexane (35:65) (isocratic); detection, UV at 260 nm; recorder chart speed, 0.25 cm/min; injection, 10 μl of a 1 μg/μl solution.

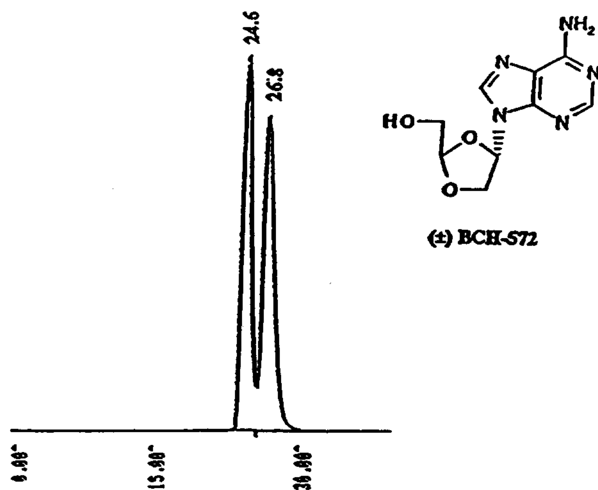

Fig. 8. Enantiomeric separation of (±)-BCH-572. Conditions as in Fig. 6, except the temperature was lowered to 15°C and the mobile phase was 0.02 *M* NaH_2PO_4–0.1 *M* NaCl (pH 7.0).

separations using just one type of chiral column. Table I gives a summary of mobile phase solutions that were used for each compound before succeeding in separating their enantiomers.

The previous dioxolane nucleosides were all separated by HPLC in the reversed-phase mode, except for one particular nucleoside [BCH-344; *cis*-2-hydroxymethyl-4- (thymin-1′-yl) -1,3-dioxolane-T], which was not successfully separated on reversed-phase chiral columns. However, as it was soluble in organic solvents, normal-phase chiral stationary phases were examined. Many normal-phase chiral columns were studied, and a cellulose column, Chiralcel OJ (250 × 4.6 mm I.D.), was found to be the most successful. Chiralcel OJ is one of a series of cellulose columns which consist of a poly-β-D-1,4 glucoside with a *p*-toluoyl ester derivative modified on the free hydroxy group [13].This makes the CSP selective for one of the enantiomers of

TABLE I

METHOD DEVELOPMENT WITH SOLVENTS FOR SEPARATIONS OF ENANTIOMERS OF BCH-187, BCH-571 AND BCH-572 USING CHIRAL α-AGP COLUMNS IN SERIES

Buffer = 0.02 *M* NaH_2PO_4 adjusted to pH 7.0 with NaOH or HCl. NR = no resolution achieved. α = separation factor, k'_2/k'_1 (k'_2 and k'_1 = capacity factors of first and second peaks, respectively.

Solvent	BCH-187	BCH-571	BCH-572
Buffer (pH 7.0)	NR	Resolution achieved, $\alpha = 1.15$	NR
CH_3CN/buffer (10:90)	NR	NR	NR
2-Propanol–buffer (8:92)	NR	NR	NR
Methanol–buffer (5:95)	NR	NR	NR
Buffer–5 m*M* TBAB (pH 7.0)	Resolution achieved, $\alpha = 1.1$	NR	NR
Buffer–5 m*M* DMOA[a] (pH 7.0)	NR	NR	NR
Buffer–0.1 *M* NaCl (pH 7.0)	NR	NR	Resolution achieved, $\alpha = 1.1$
Temperature	Ambient	Ambient	15°C

[a] DMOA = N,N,-dimethyloctylamine, 95% pure (Aldrich).

BCH-344. A baseline separation was achieved using as the mobile phase 2-propanol–*n*-hexane (35:65) giving a separation factor (α) between two resolved enantiomeric peaks [12] of about 1.5 (Fig. 9).

CONCLUSIONS

We have demonstrated a successful method development for the chiral separation of nucleoside analogues and important intermediates by HPLC. The results of biological tests [6] confirmed the importance of resolving and assessing the chiral purity of 1,3-dioxolane nucleosides. As research progresses in the development of new antivirals, we expect that concurrent development in HPLC methodology will facilitate the understanding of the impact of chirality on biological activity and host toxicity.

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