*Journal of Chromatography*, 586 (1991) 265–270
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 600

# Preparative separation and analysis of the enantiomers of [$^3$H]Abbott-69992, an HIV anti-infective nucleoside, by ligand-exchange high-performance liquid chromatography

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(First received March 20th, 1991; revised manuscript received June 27th, 1991)

ABSTRACT

Several chiral stationary phases (CSPs) were examined to separate the enantiomers of A-69992, a chiral HIV anti-infective nucleoside. The only CSP found to be effective was Nucleosil Chiral-1, a ligand-exchange CSP, which was used to prepare microgram amounts of the enantiomers of high optical purity. This appears to be the first separation of the enantiomers of a nucleoside by chiral high-performance liquid chromatography.

## INTRODUCTION

Structure–activity relationship studies have shown that the enantiomers of A-69992, an HIV anti-infective nucleoside consisting of a guanine nucleus attached to a pseudo-sugar (Fig. 1), have different antiviral activities. In order to carry out receptor-binding studies, the radiolabelled enantiomers of A-69992 of high optical purity were required. Owing, in part, to the lability of the tritium label at position 8 of the guanine residue [1,2], chiral synthesis of the pure enantiomers was deemed impractical and we sought a chromatographic method of resolving the labeled racemic mixture. A thorough search of the literature on chiral chromatography failed to reveal any examples of nucleoside separations. Thus, a screening of the chiral stationary phases (CSPs) in hand ensued. This paper describes the results of that screening and the optical resolution of [$^3$H]A-69992.

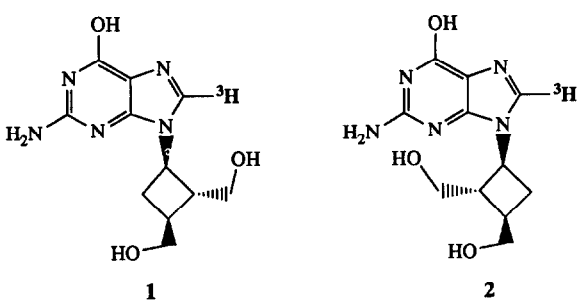


Fig. 1. The enantiomers of [$^3$H]A-69992: [$^3$H]A-75179 (**1**) and [$^3$H]A-75962 (**2**).

## EXPERIMENTAL

### *Chemicals*

Copper(II) acetate monohydrate [$Cu(OAc)_2$], triethylamine [$(C_2H_5)_3N$], glacial acetic acid (HOAc), acetonitrile ($CH_3CN$), methylene chloride ($CH_2Cl_2$), methanol ($CH_3OH$), ammonia ($NH_3$) solution, ammonium acetate ($NH_4OAc$), monobasic potassium phosphate ($KH_2PO_4$) and ethanol ($C_2H_5OH$) were purchased commercially and used without purification. All solvents except the ethanol were of high-performance liquid chromatographic (HPLC) grade. [$^3$H]A-69992 and A-75179 and A-75962 reference materials were synthesized at Abbott Labs.

*Analytical high-performance liquid chromatography*

The chromatographic mobile phase was delivered by a Perkin-Elmer Model 250 binary pump. Samples were injected using a Rheodyne Model 7125 syringe-loading sample injector with a 200-μl loop. Peaks were detected with an Applied Biosystems Model 783A UV programmable detector set at 254 nm connected in series with a Flo-One Beta Model CR radioactivity detector (Radiomatic). Chromatograms were obtained by a Mini Micro (Korea) computer and a Spectra-Physics Model SP4270 integrator. A FlAtron CH-30 column heater controlled by a TC50 controller was used for the high-temperature experiments (Table I). Analysis of optical purity was performed using a Nucleosil Chiral-1 column (250 × 4.0 mm I.D.) (Machery-Nagel, Germany). The following columns did not achieve enantiomeric resolution (see Table I): Cyclobond I (250 × 4.6 mm I.D.) (Advanced Separation Technologies, Whippany, NJ, USA), Resolvosil

TABLE I

EFFECT OF CSP, MOBILE PHASE CONCENTRATION, FLOW-RATE AND TEMPERATURE ON THE RESOLUTION AND ENANTIOSELECTIVITY OF [$^3$H]A-69992

| CSP | Mobile phase | Flow-rate (ml/min) | $t_1$[a] (min) | $t_2$[a] (min) | $R_s$[b] | $\alpha$[c] |
|---|---|---|---|---|---|---|
| Nucleosil Chiral-1 (250 × 4.0 mm I.D.) | 1 m*M* $Cu(OAc)_2$ (pH 5.75)–$CH_3CN$ (95:5) | 1.0 | 9.37 | 10.47 | 0.69 | 1.14 |
| | 0.5 m*M* $Cu(OAc)_2$ (pH 5.75)–$CH_3CN$ (95:5) | 1.0 | 9.72 | 10.90 | 0.80 | 1.22 |
| | | 0.5 | 19.40 | 21.80 | 0.80 | 1.26 |
| | 0.25 m*M* $Cu(OAc)_2$ (pH 5.75)–$CH_3CN$ (95:5) | 1.0 | 15.55 | 17.55 | 0.67 | 1.17 |
| | 0.5 m*M* $Cu(OAc)_2$ (pH 5.75)–$CH_3CN$ (95:5) at 45°C | 1.0 | 6.10 | 6.36 | 0.60 | 1.17 |
| | | 0.5 | 12.21 | 12.78 | 0.60 | 1.16 |
| | 0.5 m*M* $Cu(OAc)_2$ (pH 5.75)–$CH_3CN$ (95:5) at 38°C | 0.5 | 15.01 | 16.25 | 0.68 | 1.18 |
| Cyclobond 1 B-cyclodextrin (250 × 4.6 mm I.D.) | 50 m*M* $KH_2PO_4$ (pH 6.8) | 1.0 | 5.82 | 5.82 | | |
| Resolvosil BSA-7 (150 × 4.0 mm I.D.) | 0.1 *M* $KH_2PO_4$ (pH 6.8) | 1.0 | 1.99 | 1.99 | | |
| YMC A-KO3 (250 × 4.6 mm I.D.) | $CH_3CN$–$C_2H_5OH$ (25:75) | 1.0 | 9.60 | 9.60 | | |
| Cellulose triacetate (250 × 10 mm I.D.) | 100% $C_2H_5OH$ | 1.0 | 7.20 | 7.20 | | |

[a] $t_1$ and $t_2$ refer to the retention times of [$^3$H]A75179 (**1**) and [$^3$H]A75962 (**2**), respectively.
[b] $R_s$ is the resolution factor.
[c] $\alpha$ is the enantioselectivity factor.

BSA-7 (150 × 4.0 mm I.D.) (Machery-Nagel), YMC A-KO3 (*R*)-(+)-naphthylethylamine (250 × 4.6 mm I.D.) (YMC, Morris Plains, NJ, USA) and cellulose triacetate (250 × 10 mm I.D.) (Merck, Darmstadt, Germany).

Radiochemical purity determination was performed using a Whatman Partisil 5 ODS 3 column (250 × 4.6 mm I.D.) (Whatman, Clifton, NJ, USA).

The mobile phase used for the optical purity determination consisted of $CH_3CN$ (5%) and $Cu(OAc)_2$ (0.5 m*M*) with the pH adjusted to 5.75 with HOAc (95%). The flow-rate was set at 1.0 ml/min. The mobile phase used for the radiochemical purity determination consisted of 88% $NH_4OAc$ (0.45 *M*) plus $(C_2H_5)_3N$ (0.1%) with the pH adjusted to 4.8 with HOAc, and 12% $CH_3OH$. The flow-rate was set at 1.3 ml/min.

*Preparative high-performance liquid chromatography*

Separations were carried out using a Waters Delta Prep 3000, a Rheodyne Model 7000L syringe-loading injector with a 5-ml loop and a Waters Model 484 variable-wavelength absorbance detector operated at 254 nm. The chromatograms were obtained using a Waters Model 745B integrator. The chiral semi-preparative HPLC column used was a Nucleosil Chiral-1 (250 × 10 mm I.D.) (Machery-Nagel) made by chemically bonding L-hydroxyproline $Cu^{2+}$ complexes to Nucleosil 120 silica.

The mobile phase consisted of 5% $CH_3CN$ and 95% $Cu(OAc)_2$ (0.25 m*M*) with the pH adjusted to 5.95 with HOAc, and filtered through a 0.45-μm nylon filter. The flow-rate was set at 5.0 ml/min and the column back-pressure ranged from 950 to 1000 p.s.i. The points at which fractions were collected are shown in Fig. 4. Four fractions were collected in the first pass (group A, Table II) and three fractions in the second pass (group B: rechromatography of fraction 4A, Table II).

For sample preparation, the labeled racemic mixture was dissolved in water and diluted with mobile phase to give a concentration of *ca.* 20 μg/ml (1.0-ml injection).

*Isolation of pure enantiomers*

Fractions 1A containing purified [$^3$H]A-75179 were evaporated to dryness under reduced pressure. The residue was dissolved in the mobile phase and applied to a column of Merck silica gel (60–230 mesh) dry-packed in a 5-ml glass serological pipette. A filtered mobile phase consisting of $CH_2Cl_2$–$CH_3OH$–$NH_3$ solution (60:40:1) was used to elute the product, which was then evaporated to dryness and the residue dissolved in water. A portion was tested for the presence of copper by adding 6 *M* $NH_3$ solution. Fractions 3B containing purified [$^3$H]A-75962 were treated in an identical fashion.

## RESULTS AND DISCUSSION

Of the several CSPs tried, the enantiomers of A-69992 were resolved only on a Nucleosil Chiral-1 column. The stationary phase consists of L-hydroxyproline chemically bonded to silica gel and complexed with copper. The mobile phase contains $Cu^{2+}$ to prevent the loss of copper ion from the stationary phase. Suitably functionalized analytes compete for complexation sites and separations are based on the formation of an enantioselective ternary complex between hydroxyproline (fixed ligand),

TABLE II

CHROMATOGRAPHIC DATA FROM PREPARATIVE RESOLUTION OF [$^3$H]A-69992

[$^3$H]A-75179 is designated as **1** and [$^3$H]A-75962 is designated as **2**. Percentages given are optical purities; see text for a discussion.

| Step | Fraction 1 | Fraction 2 | Fraction 3 | Fraction 4 |
|---|---|---|---|---|
| (A) Chromatography of racemic [$^3$H]A-69992 | ~98% (**1**) | 90% (**1**)<br>10% (**2**) | 15% (**1**)<br>85% (**2**) | 9% (**1**)<br>91% (**2**) |
| Activity per fraction (μCi) | 13 | 12 | 19 | 17 |
| (B) Rechromatography of fraction 4A | 97% (**1**)<br>3% (**2**) | 6% (**1**)<br>94% (**2**) | 3% (**1**)<br>97% (**2**) | |
| Activity per fraction (μCi) | 2 | 6 | 5 | |

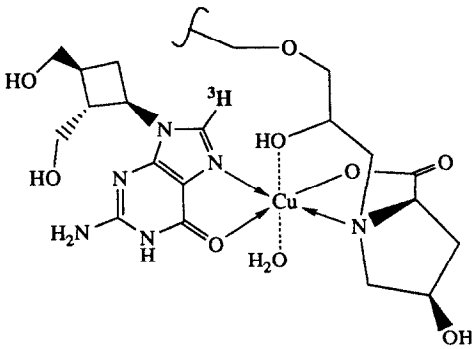


Fig. 2. Possible structure of the mixed complex between L-hydroxyproline, copper and [$^3$H]A-75179.

copper and the analyte (mobile ligand). The difference in stabilities between complexes with the D- and L-forms of the analyte leads to the separation of the enantiomers [3]. Ligand-exchange chromatography for the separation of enantiomers evolved as a result of pioneering work by Davankov *et al.* [4,5]. This method has been shown to be effective for the optical resolution of amino acids, amino acid derivatives and hydroxy acids [6–12]. Here, we report the use of ligand-exchange HPLC for the resolution of a chiral nucleoside.

It is unclear which functional groups of A-69992 are involved in chelation, but it seems likely that the guanine nucleus is participating as nucleoside copper complexes are well known [13]. Fig. 2 shows a possible structure of the mixed complex between L-hydroxyproline (bound to silica gel), copper and [$^3$H]A-75179. This structure is modeled after ligand-exchange complexes proposed for the resolution of amino acids [3,5,12]. The rationale for the structure in Fig. 2 stems from studies which show that guanine (and adenine) bind with copper at N-7 when N-9 is blocked [13] and from the fact that this portion of the nucleoside is structurally similar to α-amino acids which are thought to bind copper at the amino and carboxyl groups as shown for the L-hydroxyproline portion of the complex [3–12].

A chromatogram of the separation is shown in Fig. 3. [$^3$H]A-75179 was eluted first at 14.7 min, a

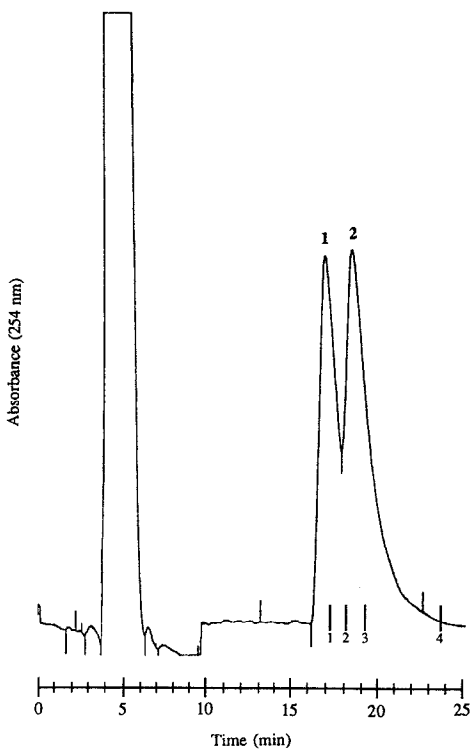


Fig. 3. Enantiomeric separation and resolution of [$^3$H]A-69992 on a Nucleosil Chiral-1 column (250 × 10 mm I.D.) showing cut-off points for fraction collection during preparative runs. The mobile phase consisted of 5% $CH_3CN$ and 95% $Cu(OAc)_2$ (0.25 m*M*) with the pH adjusted to 5.95 with HOAc. The flow-rate was set at 5.0 ml/min.

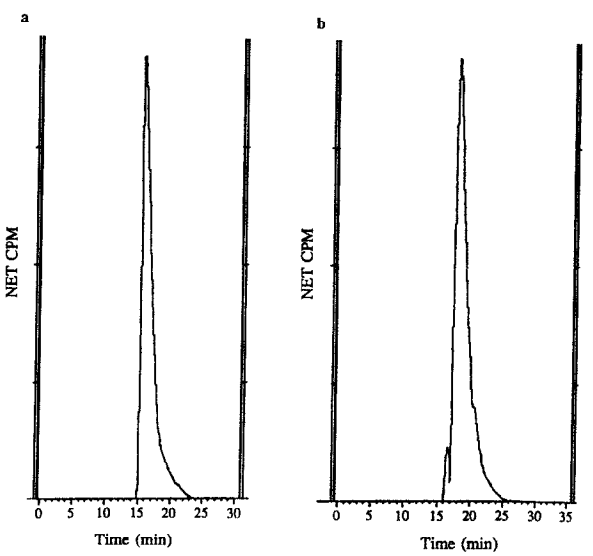


Fig. 4. Optical purity determination of (a) [$^3$H]A-75179 (**1**, *ca.* 98%), (b) [$^3$H]A-75962 (**2**, *ca.* 96%) on a Nucleosil Chiral-1 column (250 × 4.0 mm I.D.). The mobile phase consisted of 2% $CH_3CN$ and 98% $Cu(OAc)_2$ (0.50 m*M*) with the pH adjusted to 5.75 with HOAc. The flow-rate was set at 1.0 ml/min.

retention time identical with that of authentic A-75179 standard, and was followed by [$^3$H]A-75962 at 16.1 min. A number of different solvent combinations, loads and concentrations were examined during the analytical method development (Table I); however, the results could not be reproduced when done on a semi-preparative scale. This required a mobile phase consisting of 0.25 m*M* $Cu(OAc)_2$ (pH 5.95)–$CH_3CN$ (95:5) for the separation ($\alpha$ = 1.15; $R_s$ = 0.83). It should be noted that increasing the temperature of the column to 45°C, as suggested by the manufacturer, did not improve the separation. In order to obtain research amounts of [$^3$H]A-69992 enantiomers, eight 1-ml injections (75 $\mu$Ci total) of radiochemically pure racemic drug were performed and four fractions were collected for each injection (Fig. 3). The optical purities as determined by rechromatography are listed in row A of Table II. Fraction 1A was essentially pure [$^3$H]A-75179, as shown in the chromatogram in Fig. 4a. Fraction 4A was further purified by a second round of chromatography (four 1-ml injections). As detailed in row B of Table II, fraction 3B contained *ca.* 96% of [$^3$H]A-75962, as shown in Fig. 5b. The stated optical purities are only estimates owing to peak tailing and the low resolution factor. When pure unlabeled A-75179 was spiked with 2% of the other enantiomer, a shoulder was detectable (Fig. 5); however, these materials were not certified 100% enantiomerically pure so an accurate optical purity could not be determined.

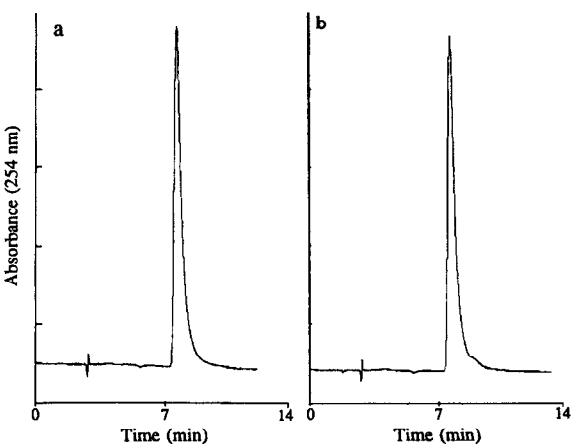


Fig. 5. HPLC of (a) A-75179 reference material and (b) A-75179 containing *ca.* 2% of A-75962.

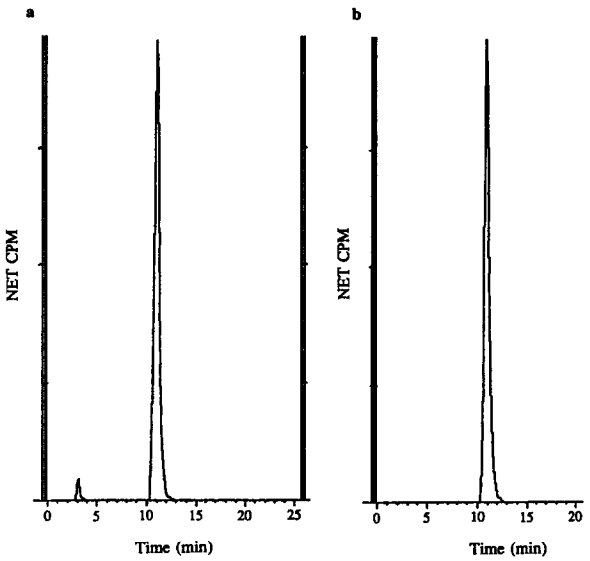


Fig. 6. Radiochemical purity determination of (a) [$^3$H]A-75179 (1, >97%) and (b) [$^3$H]A-75962 (2, >99%) on a Whatman Partisil 5 ODS 3 column (250 × 4.6 mm I.D.). The mobile phase consisted of 88% $NH_4OAc$ (0.45 *M*) plus $(C_2H_5)_3N$ (0.1%) with the pH adjusted to 4.8 with HOAc and 12% $CH_3OH$. The flow-rate was set at 1.3 ml/min.

The copper salts in fractions 1A and 3B were efficiently removed by using a gravity silica column, forming a blue band at the top of the column. A 7-$\mu$Ci amount of the [$^3$H]A-75179 and 2.6 $\mu$Ci of the [$^3$H]A-75962 were recovered. Samples were isolated *in vacuo* and each sample residue was dissolved in water. A portion of each was tested for the presence of copper salts by the addition of excess of 6 *M* $NH_3$ solution (USP XXII). The radiochemical purities were >97% and >99% for [$^3$H]A-75179 and [$^3$H]A-75962, respectively (Fig. 6).

In conclusion, the preparative procedure described above allows the isolation of microgram amounts of the enantiomers of [$^3$H]A-69992 in high purity but poor yield. This appears to be the first use of ligand-exchange HPLC for the resolution of enantiomeric nucleosides. Although attention was directed at maximization of the yield of the [$^3$H]A-75179, the yield of the [$^3$H]A-75962 could have been increased by rechromatography of mixed fractions 2A, 3A and 2B, but this was not done because the capacity factor decreased with repeated injections. Additionally, a chiral column consisting of the analogous D-hydroxyproline CSP (currently not

commercially available) would be expected to elute [$^3$H]A-75962 first, potentially increasing the yield of [$^3$H]A-75962.

ACKNOWLEDGEMENTS

The authors thank Mr. Gary Rotert for the synthesis of the radiolabeled material and Ms. Karen Wegrzyn for assistance with the manuscript.

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