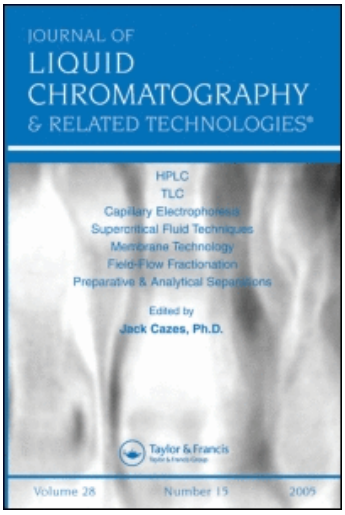


**Journal of Liquid Chromatography & Related Technologies**
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

# COMPARATIVE STUDY OF AMYLOSE AND CELLULOSE DERIVATIZED CHIRAL STATIONARY PHASES IN THE REVERSED-PHASE MODE

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Online publication date: 11 July 2000

**To cite this Article** Kazusaki, Masato , Kawabata, Hirofumi and Matsukura, Hayashi(2000) 'COMPARATIVE STUDY OF AMYLOSE AND CELLULOSE DERIVATIZED CHIRAL STATIONARY PHASES IN THE REVERSED-PHASE MODE', Journal of Liquid Chromatography & Related Technologies, 23: 18, 2819 — 2828
**To link to this Article: DOI:** 10.1081/JLC-100101234
**URL:** http://dx.doi.org/10.1081/JLC-100101234

J. LIQ. CHROM. & REL. TECHNOL., 23(18), 2819–2828 (2000)

# COMPARATIVE STUDY OF AMYLOSE AND CELLULOSE DERIVATIZED CHIRAL STATIONARY PHASES IN THE REVERSED-PHASE MODE

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## ABSTRACT

A direct, isocratic, and simple reversed-phase HPLC method was described for the separation of enantiomers, the newly synthesized potential drug substance and corresponding optical impurity, employing polysaccharide-based chiral stationary phases (Chiralpak AD-RH and Chiralcel OD-RH). A baseline separation was attained with both columns. These two chiral stationary phases exhibit opposite chiral discrimination patterns concerning the elution order of enantiomers. Chiralpak AD-RH is more suitable for resolving the enantiomers.

Low level quantification (0.05%) of the minor enantiomer is achieved. The analytical procedure was successfully applied to the detection of the optical impurity in a potential drug substance. The minor enantiomer was not determined quantitatively because of a trace level of it in the potential drug substances.

## INTRODUCTION

In the past two decades, the development of an enantioselective separation method has attracted great interest, since it became evident that the biological

activity of enantiomers is mostly restricted to one of enantiomers.[1-3] Some types of enantiomers are biologically active compounds and are called "eutomers" and then inactive ones are called "distomers." The distomers can exhibit unexpected adverse reactions, antagonistic activities, or toxic effects.

Practically, in the pharmaceutical industry, enantiomer separations by HPLC have become essential for the research and development of chiral drugs, since enantiomers of chiral drugs often exhibit marked differences in their biological activity in the living systems. As a result, marketing of chiral drugs as a single enantiomer is increasing. Therefore, it is necessary to develop analytical methods for the chiral separation to control optical purity and to gain an understanding of the clinical, pharmacological, and pharmacodynamic modes of actions, etc.



The recent significant development of high resolution columns packed with efficient chiral stationary phases (CSPs), have made a major contribution to the advancement of HPLC as a effective technique for the separation of enantiomers, without derivatization, for analysis. In the development of a HPLC method, it is usually desirable to use CSPs to directly separate the enantiomers because of the simplicity and ease of operation related to this approach. There are various types of CSPs available. Among the various CSPs, cellulose- and amylose-based CSPs have been proven to be quite versatile.

Cellulose and amylose, have a regular repeating unit of D-glucose throughout the polymers, and were used as chiral adsorbents for chromatographic enantioseparation owing to their easy availability. Native polysaccharides themselves are not practically useful chiral stationary phases in HPLC, due to their low enantioselectivities and mechanical properties. However, some modified polysaccharides show high chromatographic properties in the resolution of some racemates.[4,5]

The chiral recognition ability of polysaccharide carbamate-based CSPs appears to result from a combination of various interaction forces including hydrogen-bonding, dipole-dipole interaction, and charge transfer complex formation with the polar carbamate moiety. In addition, the sterical interaction between solute and the stationary phase seems to play a major role in the chiral recognition mechanisms of modified cellulose phases. This conformation involves the formation of a chiral groove with rigid steric requirements. Among the tris(phenyl-carbamate)derivatives of cellulose and amylose prepared so far, tris(3,5-dimethylphenylcarbamete) derivative shows quite excellent enantioselectivity for a variety of racemates.

A wide range of cellulose-based CSPs are now available for the separation of enantiomers by HPLC and they have proved to be useful in the chromatographic resolution of racemic compounds. However, most of these columns

were used in the normal-phase mode. With the background of the advancement of industrial technologies, some chiral columns for reversed-phase mode are commercially available.[6]

Reversed-phase chromatographic techniques are preferable in pharmaceutical analysis to normal phase chromatographic techniques, since they involve the use of less organic solvents and do not use environmentally harmful solvents, such as hexane, chloroform, and methylene chloride. Besides, many drugs exist as salts which are water soluble and are easier to prepare samples for the reversed-phase chromatography. Aqueous eluents are particularly useful for investigating the pharmacokinetics and pharmacodynamics of chiral drugs in living systems.

*R*-2-(4-bromo-2-fluorobenzyl)-(1,2,3,4-tetrahydropyrrolo[1,2-a]pyrazine-4-spiro-3'-pyrrolidine)-1,2',3,5'-tetrone possessing aldose reductase inhibitory activities has been synthesized as a potential drug substance for the treatment of diabetic complications, such as cataract formation, retinopathy, neuropathy, and nephropathy,[7] and developed as a single enantiomer because it shows stronger activity than the *S*-enantiomer. The chemical structure is shown in Figure 1.



The aim of this work is to develop a method for determining the optical purity of the potential drug substance. Very often, in addition to achieving the separation of enantiomers, it is also desirable to elute the minor enantiomer before the major one to avoid possible interference caused by the tail of the major enantiomer. In this study, it was found that the elution order of the enantiomeric pair was reversed by changing the stationary phase from cellulose-

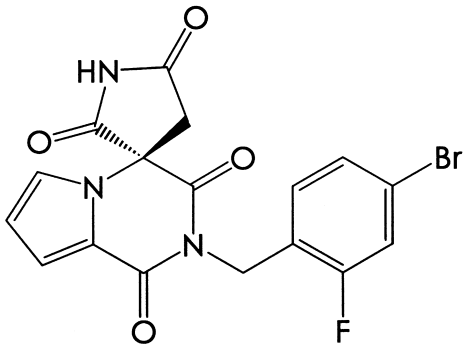


**Figure 1**. Chemical structure of *R*-2-(4-bromo-2-fluorobenzyl)-(1,2,3,4-tetrahydropyrrolo[1,2-a]pyrazine-4-spiro-3'-pyrrolidine)-1,2',3,5'-tetrone.

based phase to an amylose based one. This observation could be useful for method development.

This paper describes the HPLC separation of optical isomers on the amylose and cellulose derivatized-columns through the modification of the mobile phase. Finally, this separation method was applied to the optical purity testing for quality control of potential drug substances as a single enantiomer.

## EXPERIMENTAL

### Chemicals and Reagents

*R*-2-(4-bromo-2-fluorobenzyl)-(1,2,3,4-tetrahydropyrrolo-[1,2-a]-pyrazine-4-spiro-3'-pyrrolidine)-1,2',3,5'-tetrone and corresponding *S*-enantiomer were synthesized in the laboratory of Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan). Ammonium acetate was of reagent grade from Wako Pure Chemical Industries Ltd. (Osaka, Japan). HPLC grade acetonitrile was also purchased from Wako Pure Chemical Industries Ltd. The water was deionized prior to usage.



### Apparatus

The HPLC system consisted of a solvent delivery system, column oven, variable wavelength detector set at 297 nm at a range of 0.032 AUFS, and variable volume injector with a set injection volume of 10 μL (all Hitachi model D-7000 system, Hitachi, Tokyo, Japan). Chiral columns, amylose tris(3,5-dimethylphenylcarbamate) column, and cellulose tris(3,5-dimethylphenyl-carbamate) column, known as Chiralpak AD-RH and Chiralcel OD-RH, respectively, (both 150mm x 4.6 mm i.d., 5 μm particle, Daicel Chemical Industries, Ltd., Tokyo, Japan) were maintained at 40°C. Resolution was carried out with an acetonitrile - 0.01 M acetate buffer (pH 4.6) mixture. The flow rate was 0.5 mL/min.

## RESULTS AND DISCUSSION

### Chromatography

Enantiomers were completely separated on two types of columns, derivatized amylose and cellulose chiral columns, respectively, these structures of repeating unit being depicted in Figure 2. As shown in Figure 3, retention would result primarily from conventional solute-bonded phase interactions (i.e., decreasing retention factor with increasing organic character of the mobile

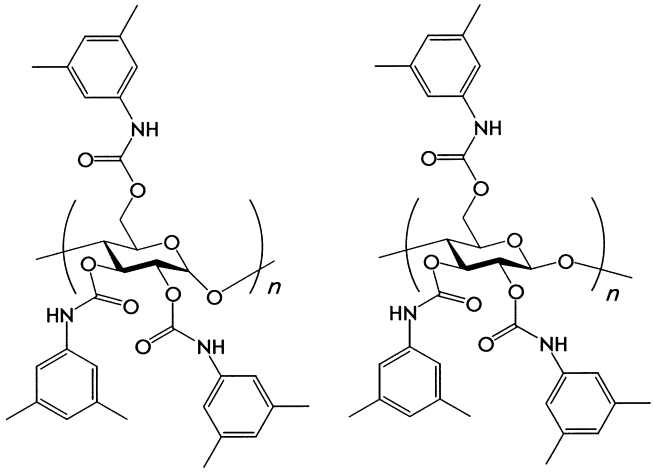


**Figure 2**. Chemical structures of amylose and cellulose derivative.



phase). It has been assumed, that the separation of enantiomers on the amylose- and cellulose-based CSPs was due to the formation of solute-CSP complexes between the enantiomers and the chiral cavities in the higher order structures of the CSPs. *R*-enantiomer was eluted prior to *S*-enantiomer on the cellulose-based column.

However, in the case of the amylose-based column, the order of elution was reversed. This fact indicates that these two stationary phases are complementary in recognizing chirality. Okamoto's research group has reported numerous examples in which Chiralcel OD and Chiralpak AD columns showed different chiral recognition abilities, as well as different elution orders of many enantiomeric pairs on the two columns.[8,9]

Since the derivatization group on both CSPs was the same, the different retention behaviors of the two CSPs should be due only to the conformational difference between the two CSPs. This suggests, that the retention of solutes not only depends upon the derivatization groups, but also depends upon the supramolecular structure of the CSPs. It is a well-established fact that the supramolecular organization of molecular systems influences their physicochemical properties. In other words, this difference in selectivity may be attributed to a high-order structural difference between derivatized cellulose and amylose.

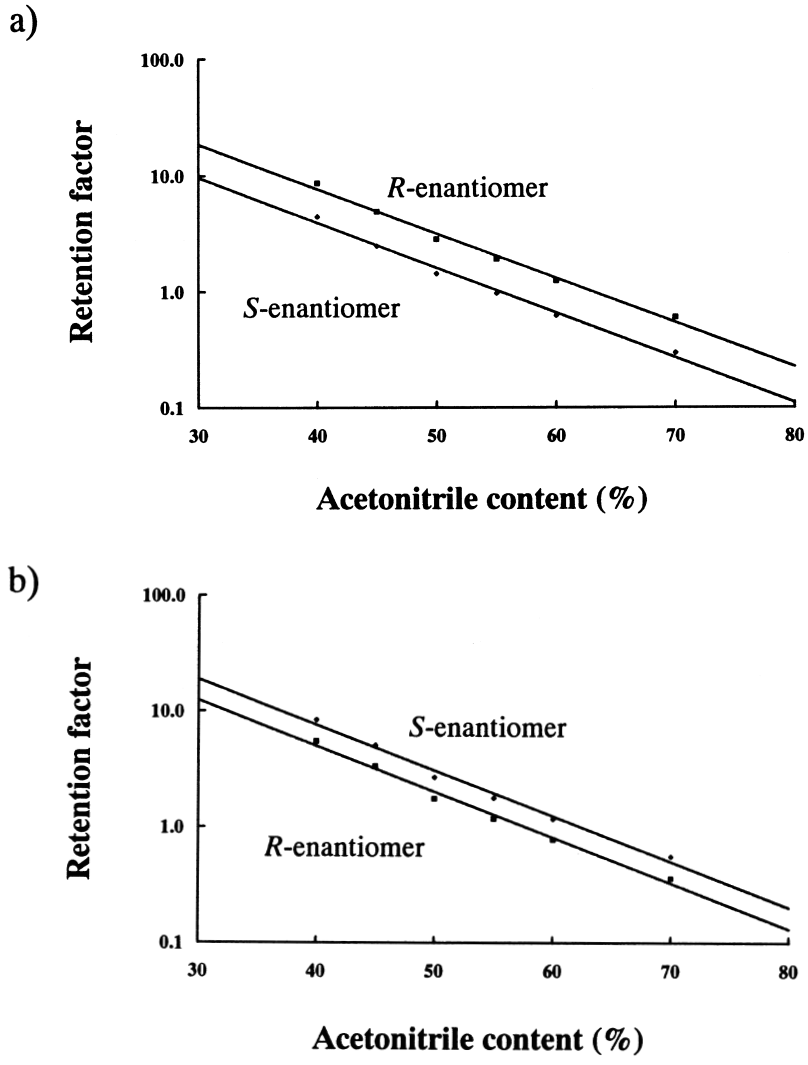


**Figure 3**. Retention of enantiomers on (a) the amylose-based column and (b) the cellulose-based column. HPLC operating conditions: column, (a) Chiralpak AD-RH and (b) Chiralcel OD-RH (250mm x 4.6mm i.d.); mobile phase, a mixture of acetonitrile and 0.01 *M* acetate buffer (pH4.7); detection, UV at 297 nm; column temperature, 40°C; flow rate, 0.5 mL/min.

Possible structures are left-handed 3/2 and 4/1 helical chain conformations for tris(phenylcarbamate) of cellulose and amylose, respectively. These conformations are attributable to the linkage of β(1→4) type for cellulose units and α(1→4) type for amylose. This structural difference seems to be responsible for the fact that the *R*-enantiomer has stronger interaction than the *S*-enantiomer on an amylose-based column, and *vice versa* on a cellulose-based column. This observation can be a useful guide for future method development in which a reversal of elution order is desired.

The separation factor of a racemic mixture obtained with the amylose-based column was better than that obtained with the cellulose-based column, as shown in Figure 4. Interestingly, the enantioselectivity is essentially unchanged over the entire range of acetonitrile content. This suggests that the conformation of the polymeric phase are not affected by acetonitrile content in the mobile phase, and that the interaction on the basis of hydrogen-bonding is not the main driving force for enantioseparation.



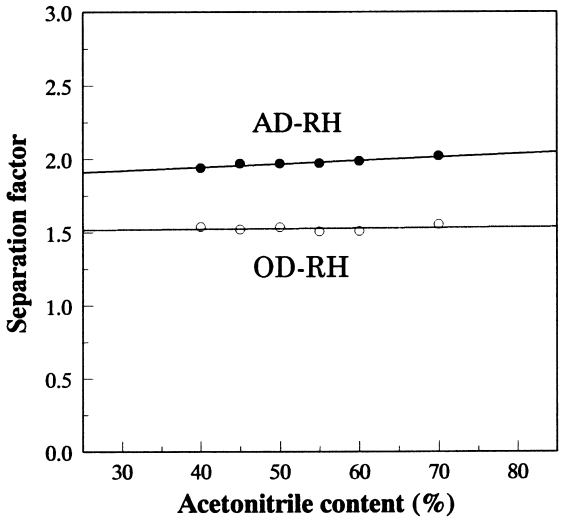


**Figure 4**. Influence of the acetonitrile content in the mobile phase on the separation between enantiomers on the amylose and cellulose-based columns. HPLC operating conditions: column, Chiralpak AD-RH and Chiralcel OD-RH (250mm x 4.6mm i.d.); mobile phase, a mixture of acetonitrile and 0.01 *M* acetate buffer (pH4.7); detection, UV at 297 nm; column temperature, 40°C; flow rate, 0.5 mL/min.

**Analytical Figure**

Experiments were performed to obtain a mobile phase which will give the optimum separation and selectivity on the amylose-based column, because elution of *S*-enantiomer prior to *R*-enantiomer makes this method ideal for trace analysis of the *S*-enantiomer present in the *R*-enantiomer. A mixture of acetonitrile and acetate buffer (1:1) was used as the mobile phase in consideration of the properties of the analyte and the column.

Retention times of *R*- and *S*-enantiomers were 8.6 min and 13.2 min, respectively. Retention factors ($k$) for *R*- and *S*-enantiomers were 1.3 and 2.5, respectively. The calculated theoretical plates for *R*- and *S*-enantiomers were 2000 and 2400 per 15 cm column. Relative retention of *R*- and *S*-enantiomers, as expressed by separation factor, $\alpha$, was calculated to be 1.9. Resolution (Rs) between *R*- and *S*-enantiomer peaks was 4.9.



Some validations, such as linearity, accuracy, and precision were also demonstrated. The amount of the *S*-enantiomer present in the *R*-enantiomer was quantified by peak area. The responses of each enantiomer from the UV detector was linear from 5 to 25 ng injections. Typical regression line equations of racemates both have correlation coefficients of more than 0.998 with negligible $y$-intercepts, and essentially pass through the origin, respectively.

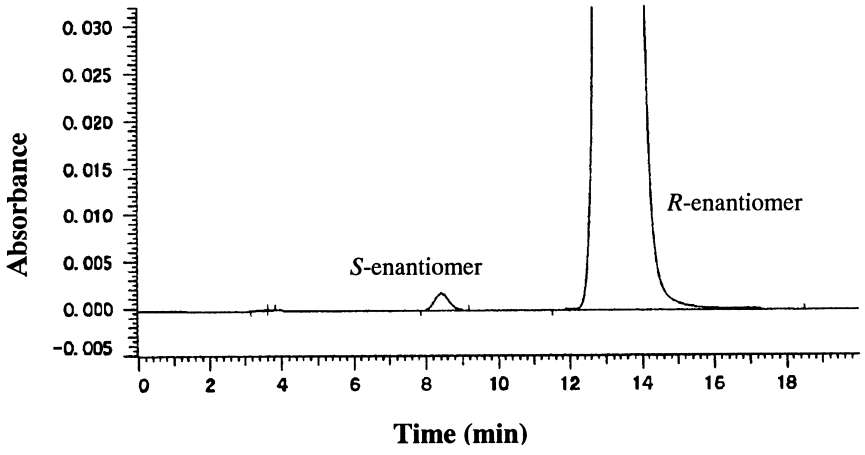


**Figure 5.** Low level detection (0.1%) of *S*-enantiomer (optical impurity) spiked in *R*-enantiomer (potential drug substance). HPLC operating conditions: column, Chiralpak AD-RH (250mm x 4.6mm i.d.); mobile phase, a mixture of acetonitrile and 0.01 M acetate buffer, pH4.6 (1/1, v/v); detection, UV at 297 nm; column temperature, 40°C; flow rate, 0.5 mL/min.

**Table 1**

**Results of Optical Purity Testing for Bulk Product**

| Lot No. | Optical Impurity Content (%) |
|---|---|
| A | N.D. |
| B | N.D. |
| C | N.D. |
| D | N.D. |
| E | N.D. |

N.D.: not determined. Limit of quantification, 0.05%.



Good linearity on this method was confirmed. The lowest quantification level was 5 ng of the amount of the *S*-enantiomer injected into HPLC with the scattering of 3% relative standard deviation. This quantification level corresponds to 0.05% related to the amount of the bulk product of 10 μg injected into HPLC. Standard addition and recovery experiment of *S*-enantiomer into *R*-enantiomer was carried out at a level of 0.1% to determine the accuracy and precision of quantification of optical impurity in potential drug substance. A chromatogram showing the detection of *S*-enantiomer at the 0.1% level is shown in Figure 5. The recovery rate of *S*-enantiomer averaged 99.8% with 2.7% relative standard deviation. Good accuracy and precision was confirmed.

Finally, the determination of *S*-enantiomer in potential bulk products was performed using the proposed method. The results of the determination of *S*-enantiomer (optical impurity) in bulk products are summarized in Table 1. *S*-enantiomer was not determined due to the trace level of less than 0.05% in the potential bulk product.

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Received February 1, 2000 Author's Revisions July 8, 2000
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