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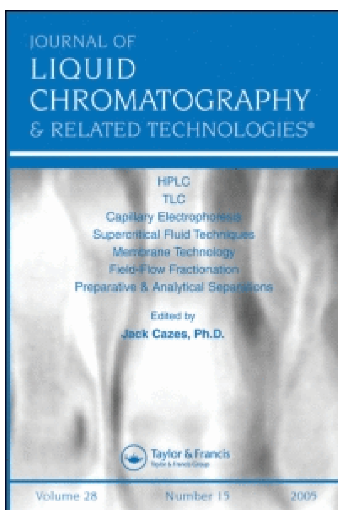


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# DETERMINATION OF ENANTIOMERS IN HUMAN SERUM BY DIRECT INJECTION ONTO A β-CYCLODEXTRIN HPLC BONDED PHASE

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## ABSTRACT

A method is proposed for the direct enantiomeric analysis of chiral drugs in human serum by HPLC. The method is based on a commercially available β-cyclodextrin bonded phase which incorporates diol as well as cyclodextrin moieties. The proteinaceous components of the serum, which are shown to elute with the void volume, are well separated from the enantiomeric analytes. This allows for the rapid determination of the enantiomeric composition of the drugs.

## INTRODUCTION

The analysis of biologically derived samples present unique analytical challenges. Chromatographic analysis requires sample compatibility with both the mobile phase and the stationary phase. One type of phase which has been notably applied to the direct analysis of biological samples has been the diol-type phase[1,2]. The diol functionality mitigates the deleterious effects of the anionic silica surface without exposing proteins in the sample to the excessive hydrophobic interactions prevalent on the reversed-phase sorbents which contribute to denaturation of the proteins.



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The current interest in the resolution of optical isomers is due to the growing awareness of the role that configuration plays in biological activity[3]. Although chiral separations may be achieved by derivatizing the enantiomers with optically pure derivatizing agents, much of the work in chiral separations has been directed to the development of new chiral stationary phases (CSP). Some of the problems associated with the use of chiral derivatizing agents include availability of optically pure derivatizing agent, chiral discrimination in the derivatizing reaction, lack of suitable functionality for derivatization in the analyte, possibility of racemization under the reaction conditions and additional validation required for the methodology[4].

A tremendous amount of effort has been focussed on analysis of "clean" or nonbiologically derived samples. For detailed *in vivo* pharmacologic profiling of isomeric drugs, the direct resolution of optical isomers in biological fluids is highly desirable because of the reduced sample preparation time and reduced probability of enantiomeric perturbation induced by sample clean-up. Unfortunately, many of the CSP currently available, including the Pirkle-type and derivatized cellulosic phases, require mobile phases which are incompatible with the direct injection of biologically derived samples.

Among the most successful of the CSP have been the cyclodextrin-based phases which have been used for enantiomeric separations of a wide variety of compounds including drugs[5,6], alkaloids[7], crown ethers[8] and derivatized[9,10] as well as underivatized amino acids[11]. In most cases, the enantiomeric separations obtained were of the underivatized compound and were accomplished under reversed-phase conditions.

Two approaches have been used to attach the cyclodextrin to the silica substrate. One approach is to synthesize a modified cyclodextrin which incorporates an active silane functionality that is subsequently reacted with the silica[12]. The advantage of this approach is that it produces a bonded phase which contains only the bonded cyclodextrin moiety. The other approach involves binding the cyclodextrin through a spacer which has been previously attached

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to the silica[13]. The advantages of this approach are that the linkage between the spacer and the cyclodextrin is hydrolytically stable and the spacer introduces a minimal amount of additional nonstereospecific interactions with a complexed analyte.

Haginaka and Wakai[14] recently reported a new cyclodextrin bonded phase based on a variation of the first method in which a cyclodextrin-carbamoylated triethoxysilane was reacted with silica that had been previously modified with (3-glycidoxypropyl)trimethoxysilane. The (3-glycidoxypropyl)silane was used to introduce a diol moiety onto the silica substrate. The mixed-phase that they produced was found to be effective for the direct analysis of drug enantiomers in human serum. A commercially available cyclodextrin CSP is prepared using the second approach and employs a 3-glycidoxypropylsilane as the spacer. The purpose of our study was to see if the cyclodextrin-based phases synthesized according to the second method[13] could be used analogously for the direct injection analysis of drug enantiomers in human serum. It was thought likely that the size of the cyclodextrin might result in less than total coverage of the spacer moietites thereby producing a mixed-phase, combining diol and cyclodextrin moieties, which would be compatible with serum proteins. Bonding experiments were conducted to establish the relative surface concentration of spacer and cyclodextrin moieties. However, chromatographic experiments were conducted with commercially available columns to establish the viability of the approach.

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## EXPERIMENTAL SECTION

### Reagents

(3-Glycidoxypropyl)trimethoxysilane was obtained from Petrarch Systems (Bristol, PA). The $\beta$-cyclodextrin was obtained from Advanced Separation Technologies, Inc. (Whippany, NJ). HPLC grade acetonitrile, water and the phosphate buffer were obtained from Fisher Scientific. The drug samples were obtained as methanolic solutions from Sigma Chemical Company (St. Louis, MO).

TABLE I.
LIST OF BONDED SORBENTS

| Source | surface area ($m^2/g$) | pore size (Å) | $\%C^1_{spacer}$ | $\%C^2_{CD}$ | $\mu mol/m^2$ (spacer) | $\mu mol/m^2$ (CD) |
|---|---|---|---|---|---|---|
| Nucleosil | 100 | 300 | 1.44 | 2.92 | 2.1 | 0.3 |
| Nucleosil | 100 | 300 | 1.64 | 2.55 | 2.4 | 0.3 |
| Hypersil | 170 | 100 | 3.22 | 5.49 | 2.9 | 0.2 |
| Spherisorb | 220 | 80 | 2.62 | 4.96 | 1.8 | 0.2 |
| Spherisorb | 220 | 80 | 3.24 | 4.93 | 2.2 | 0.1 |
| Spherisorb | 190 | 300 | 2.59 | 5.33 | 2.0 | 0.3 |

1 %C due to the spacer used to link the CD to the silica substrate

2 %C due to the spacer linkage plus the CD

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**Bonded phases**

Several different batches of cyclodextrin bonded phases were prepared on silica obtained from various sources. The bonded phases were prepared using the two-step procedure developed by Armstrong[13]. Because it was assumed that the spacer ligand epoxides would be hydrolyzed under the acidic chromatographic mobile phase conditions used, no specific procedure was used to hydrolyze the residual epoxides that may remain on the silica surface subsequent to the addition of the cyclodextrin moiety. Bare silica, silica modified with the linkage chain, as well as the cyclodextrin bonded silica were all submitted for carbon analysis (Galbraith Laboratories, Inc., Knoxville, TN). The pertinent bonding results are listed in Table I. The surface concentration was calculated according to the equation[15]

$$\textit{surface concentration}\ (\mu mol/m^2) = \frac{\%C \cdot 10^6}{1200N_c - \%C(M-1)} \cdot \frac{1}{S}$$

where %C is the percent carbon (w/w) obtained from elemental analysis, $N_C$ is the total number of carbons in the bonded ligand, S is the surface area of the silica and M is the molecular weight of the bonded ligand.

## Chromatographic Apparatus

The chromatographic experiments were performed using a Shimadzu LC-600 Liquid Chromatograph. Chromatographic retention data was acquired with a C-R3A Chromatopac Data System. The chromatographic columns (received from Advanced Separations Technology, Inc., Whippany, NJ) were 250 x 4.6 mm i.d. stainless steel packed with 5 μm Cyclobond I (β-CD). Detection was accomplished using a Shimadzu SPD-6A variable wavelength detector set at the appropriate wavelength. Undiluted human serum and spiked serum samples (20 μL) were injected directly onto the HPLC system.

The mobile phase used was acetonitrile/phosphate buffer. Acetonitrile concentrations varied from 1 to 10%. The pH range was from 4 to 6.9. A silica presaturator column was placed in-line, prior to the injector, to extend the life of the silica-based β-cyclodextrin column.

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## Fluid Samples

Human serum (venous samples) were collected by standard venipuncture technique into Vacu-tainer tubes without additives, clotted and centrifuged. The supernatant serum samples were removed and stored at 0°C until analysis. The samples were spiked with the appropriate volumes of standard methanolic solution.

# RESULTS AND DISCUSSION

## Bonding Results

As can be seen from Table I, in all cases the spacer is present in much higher concentrations than the cyclodextrin bonded moiety. Indeed, in almost all cases, the spacer is

TABLE II.
CHROMATOGRAPHIC DATA FOR THE DIRECT DETERMINATION OF THE ENANTIOMERIC COMPOSITION OF CHIRAL DRUGS IN HUMAN SERUM

| Compound | $k'^{1}$ | $\alpha$ | $R_s$ | Mobile Phase |
|---|---|---|---|---|
| chlorpheniramine maleate | 5.11[2] | 1.07 | 0.84 | 10% acetonitrile/buffer (100mM phosphate, pH 4.2); 0.8 mL/min |
| chlorthalidone | 1.93[2] | 1.28 | 2.02 | 10% acetonitrile/buffer (100mM phosphate, pH 4.2); 0.8 mL/min |
| hexobarbital | 6.08[3] | 1.15 | 1.39 | 5% acetonitrile/buffer (100mM phosphate, pH 6.9); 0.8 mL/min |
| mephobarbital | 6.27[3] | 1.12 | 1.10 | 5% acetonitrile/buffer (100mM phosphate, pH 6.9); 0.8 mL/min |

1 capacity factor of the first eluted enantiomer

2 two 25 cm x 4.6 mm I.D. Cyclobond I columns used in series

3 one 25 cm x 4.6 mm I.D. Cyclobond I column used

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present in roughly an order of magnitude greater concentration than the cyclodextrin moiety. It is reasonable to assume that although excess cyclodextrin is used, the size of the cyclodextrin which is much bigger than the spacer, results in the cyclodextrin being somewhat excluded from the silica pores, thereby preventing total coverage of the spacers. This conclusion is supported by the fact that the highest surface concentrations of cyclodextrin are obtained on the large pore silica substrates. Simialr results have been previously reported by Stalcup, et al[16]. As a consequence, the stationary phase produced using this method is a mixed-functionality stationary phase which incorporates both cyclodextrin as well as unreacted spacer moieties. It has been reported that for nonenantiomeric and normal phase separations, that the cyclodextrin phases behave somewhat like a diol phase[17]. This is not too surprising considering the reduced surface concentration of cyclodextrin moieties relative to the surface concentration of spacer ligands.

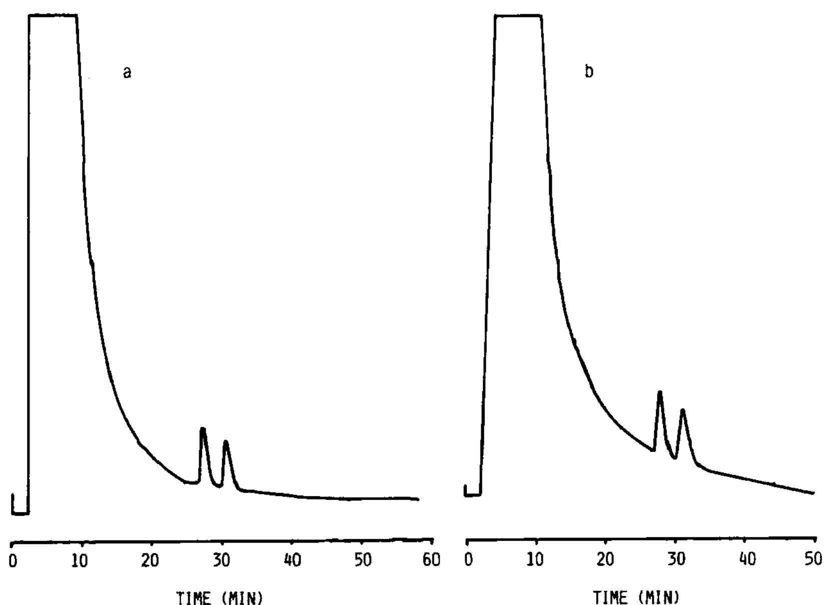


Figure 1. Chromatographic separation of hexobarbital enantiomers on a 25 cm x 4.6 mm I.D. Cyclobond I ($\beta$-cyclodextrin) column. Experimental conditions given in Table II. (a) after 20 serum injections; (b) after 60 serum injections. Sample spike was 50 $\mu$g hexobarbital/mL serum.

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**Chromatographic results**

Table II lists the chromatographic data for several other drugs which were spiked into serum. In all cases, the analyte peaks were well resolved from the serum protein. Typical chromatograms obtained using this method are illustrated in Figure 1 (hexobarbital spiked into human serum). As can be seen from the chromatograms, the serum proteins elute with the void volume while the smaller, chiral analytes are retained. The retention of the chiral analytes is no doubt due to interaction with the cyclodextrin through inclusion complexation to effect the enantiomeric separation. The hexobarbital peaks are well separated from the protein peaks and readily quantitated. Figure 1a shows the analysis after 20 injections; Figure 1b shows the same

analysis after 60 injections. Over one hundred direct injections of human serum were made onto the $\beta$-cyclodextrin phase with no deleterious effects on the chromatography.

## CONCLUSION

The commercially available $\beta$-cyclodextrin column has been found to be applicable to the direct analysis of enantiomers in human serum. The results of this study indicate that other cyclodextrin-based phases synthesized using the same protocol, including the hydroxypropyl-$\beta$-cyclodextrin as well as the $\alpha$-and $\gamma$-cyclodextrin phases, may also be amenable to direct determination of the enantiocomposition of chiral analytes in serum. In addition, these results also imply that cyclodextrin-based phases may have some utility for more routine (nonstereospecific) bioanalysis because of the compatibility of mobile phases used with cyclodextrin and reversed phase ($C_8$ or $C_{18}$) HPLC columns (coupled column or multi-dimensional chromatography). The direct analysis of biological fluids greatly decreases the analysis time required as well as improves the overall accuracy of the determination.

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