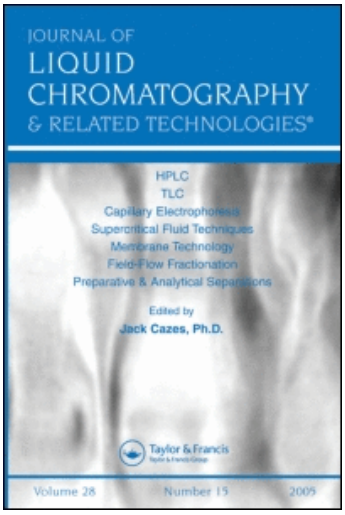

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COMPARISON OF TWO METHODS OF PREPARATION OF THE STATIONARY PHASE FOR HPLC CHIRAL COLUMNS BASED ON *tris*(3,5-DIMETHYLPHENYLCARBAMOYL) CELLULOSE

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ABSTRACT

Stationary phase based on *tris*(3,5-dimethylphenylcarbamoyl) cellulose is prepared under various conditions (precipitation, evaporation, sieving), and examined in HPLC columns for enantioseparation of racemic mixtures 1-10. Number of theoretical plates (N) and separation parameters (α and R_S) are compared with those obtained with commercially available column based on the same polymeric material (*Chiralcel OD*, from Daicel Co.). Higher efficiency of the columns prepared by precipitation under specific conditions and followed by sieving of stationary phase, over those prepared by evaporation and (or without) sieving, is demonstrated.

INTRODUCTION

Determination of enantiomeric purity, monitoring of the procedure for preparation of enantiomerically pure compounds, preparative separation of enantiomers, and determination of differences in biological response for certain enantiomers have the great role in modern pharmaceutical, agrochemical, food and chemical industry.[1,2] In this framework, chromatographic methods became indispensable thanks to their relative simplicity, high accuracy, and fast accumulation of the experimental results.[3,4] HPLC columns with chiral stationary phases (CSPs) have been developed and are currently available on the market. Polysaccharide-based, and in particular cellulose-based CSPs, have proved extremely successful for the separation of a wide range of chiral analytes.[5,6]

Several studies have been devoted to test cellulose derivatives coated on macroporous silica for the development of new CSPs[7-10] but little attention has been paid on the influence of the preparation conditions of the CSP on its performance. The coating on the silica may be achieved by evaporation of a solution of the cellulose derivative in an organic solvent as well as by precipitation of the cellulose derivative by slow addition of a proper non-solvent. The comparison between these two methods has been performed in the case of trisphenylcarbamoyl cellulose[11] and *meta*-methylbenzoyl cellulose.[10] In the former, the chromatographic results revealed no distinction between these two methods, but in the latter the chiral discrimination ability depended to a great extent on the coating method. In particular precipitation method produced a more efficient CSP than evaporation one. In several papers sieving of the CSP before column packing is not mentioned and, when it is mentioned,[12-14] the influence of sieving is not discussed in detail.

Among the different cellulose derivatives, *tris*(3,5-dimethylphenyl-carbamoyl) cellulose[15] is particularly versatile. Of 510 racemates tested, 229 were completely resolved and 86 partially resolved with this cellulose derivative supported on silica.[16,17] The mechanism for chiral recognition on this CSP has been hypothezed.[18-19] It has been proposed that hydrogen-bonding plays a strong role in the selective chiral interaction process and this interaction can occur at two potential sites of the carbamate functionality.

In spite of the extensive use of *tris*(3,5-dimethylphenylcarbamoyl) cellulose coated on silica as CSP (commercially available under the names *Chiralcel OD* by Daicel, Japan and *Pharmachir 4C* by A.I.T., France) to our knowledge, no studies of the effect of the preparation conditions of this CSP on its performance have been reported. The evaporation[9,14,15] and precipitation[20] methods are separately reported in literature but not directly compared.

This fact largely stimulated the present investigation aimed at comparing the resolving ability of CSPs based on *tris*(3,5-dimethylphenylcarbamoyl) cellulose prepared under different conditions. The results of separation have been compared with those obtained with commercially available columns based on the same polymeric material (*Chiralcel OD* by Daicel, Japan).

EXPERIMENTAL

Chemicals

Ten racemic compounds were used for chiral testing of HPLC columns (Figure 1). Racemic trans-stilbene oxide (1), flavanon (2), Trögers base (3), benzoin methyl ether (4), benzoin (5), and phenyl ethyl amine (10) were purchased from Sigma-Aldrich (Aldrich Chimica, Milano, Italy). Racemic ibuprofen phenyl amide (6) was prepared from 4-isobutyl-α-methyl-phenylacetic acid (Sigma-Aldrich) and aniline. Phenyl amide phenyl ethyl amine (8) and 1-naphthyl amide phenyl ethyl amine (9) were synthesized from phenyl ethyl amine and benzoyl or 1-naphthoyl chloride, respectively. Preparation procedure will be published elsewhere for benzodiazepine (7).

Microcrystalline cellulose (Avicel) was purchased from Merck (Darmstadt, Germany), 3,5-dimethylphenyl isocyanate from Lancaster (Mühlheim/Main, Germany), 3-aminopropyltriethoxy silane from Aldrich, and HPLC silica gel Nucleosil 1000-7 from Macherey-Nagel (Düren, Germany).

n-Hexane and isopropanol used for HPLC chromatography were analytical grade from J. T. Baker B.V. (Deventer, Holland) and redistilled before using to obtain HPLC quality. Acetone and pyridine used in reactions were analytical grade.

Apparatus

^{1}H-NMR spectrum was recorded with a Bruker AC 200 (200.13 MHz) spectrometer. FTIR spectrum was taken on a Perkin-Elmer 1750 spectrometer in KBr pellet.

Chromatography was performed with a Knauer WellChrom Maxi-Star K-1000 pump (Knauer GmbH, Berlin, Germany) using a Knauer HPLC 6-port-valves injector with a 20 μL loop. Detection was achieved at 254 nm with a Knauer WellChrom K-2500 detector. Integration of the chromatograms was made with the BDS software package (Barspec Ltd., Rehovot, Israel).

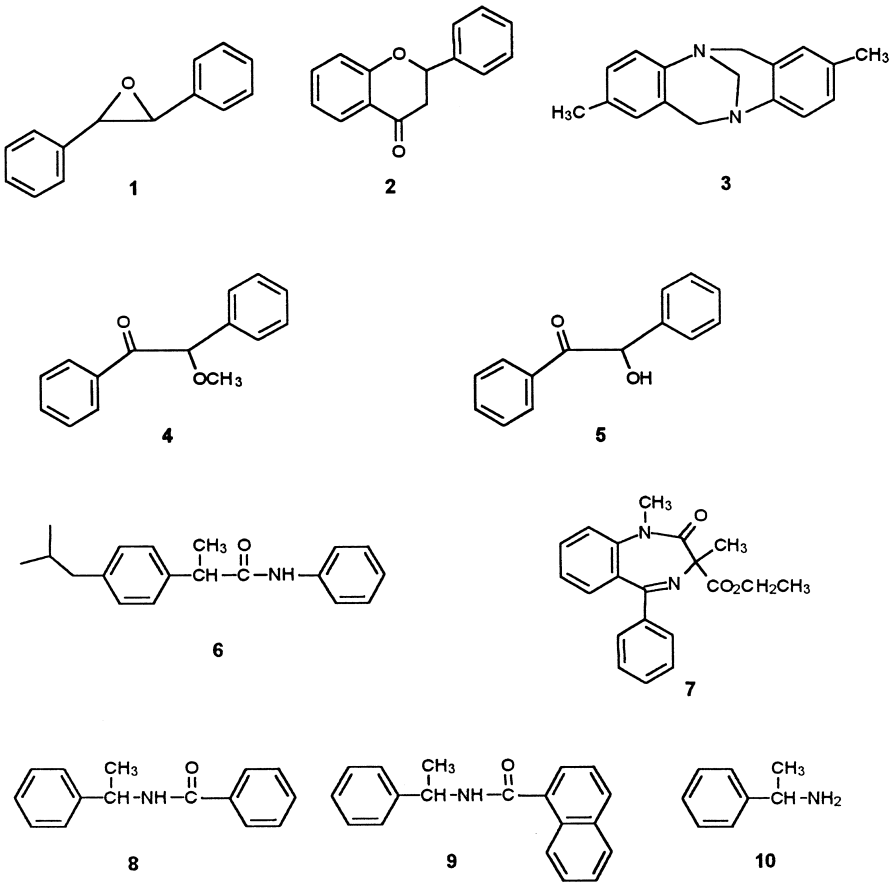

Figure 1. Racemic compounds used for evaluation of HPLC columns.

The following parameters were measured:

k_1': capacity factor of the first eluted enantiomer, $(t_1-t_0)/t_0$;
k_2': capacity factor of the second eluted enantiomer, $(t_2-t_0)/t_0$;
α: selectivity factor, $\alpha = k_2'/ k_1'$;
R_S: resolution factor, $R_S = 2(t_2-t_1)/(w_1+w_2)$; w is the base width of the peaks.

The packing of HPLC columns was performed by a slurry technique using a Knauer pneumatic HPLC-pump.

Preparation of Stationary Phase

Tris(3,5-dimethylphenylcarbamoyl) cellulose (CS-1) was prepared as follows: the dried cellulose was poured into dry pyridine and 4.5 equivalents of 3,5-dimethylphenyl isocyanate were added. The mixture was maintained at 80°C for 16 h under inert atmosphere (N_2). The resulting solution was allowed to cool to 25°C and dried under vacuum.

The crude solid was dispersed in dichloromethane and the product was recovered by precipitation into 3 volumes of methanol. The recovered white solid was filtered, washed well with methanol, and dried under vacuum at 50°C. Yield was 90%. ^{1}H-NMR and FTIR spectra indicate the complete conversion of hydroxy groups into carbamates

Evaporation method

Cellulose derivative (2.50 g) was dissolved in acetone (100 mL) and added to 10.0 g of silanized silica gel previously wetted with acetone. After 0.5 hour of magnetic mixing solvent was slowly evaporated under reduced pressure (50 mm Hg). The HPLC column filled with this material was named A-unsieved. The powder (5.75 g) was wet sieved under n-hexane/isopropanol (90/10) through a 28 μm sieve (Endcotts Ltd., England).

The collected suspension was decanted and the supernatant was discarded. The sieved powder (3.11 g) was then recovered after exhaustive evaporation of residual solvent and used to pack an HPLC column named A-sieved.

Precipitation method

Previously silanized silica gel (10.0 g) was suspended in the solution of 2.50 g of CS-1 in 100 ml of acetone. During vigorous magnetic stirring 700 mL of n-hexane (HPLC grade) were added to complete precipitation. When the addition was complete (6 hours), the product was filtered and residual solvent evaporated by rotavapor.

Solid material was suspended in acetone (50 mL), sonicated for 5 min, and acetone evaporated. The HPLC column filled with this material was named B-unsieved.

Coated material obtained as above (7.0 g) was sieved through a 28 μm sieve and with the sieved powder (6.1 g) the two HPLC columns, named B-sieved, were packed. Both of the columns have the same characteristics.

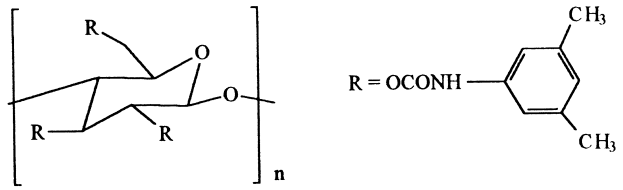

Figure 2. Chemical structure of the *tris*(3,5-dimethylphenylcarbamoyl) cellulose (CS-1).

Table 1

General Characteristics of the Tested Columns, as Determined for Benzene Using n-Hexane/Isopropanol (9:1) as Eluent

Column	t_0/min/	N
A-unsieved	5.9	1720
A-sieved	5.8	7780
B-unsieved	5.9	4020
B-sieved	6.0	8370
Chiralcel OD	5.9	8465

Column Packing

The packing of HPLC columns was performed by the slurry method with a stationary phase (3.0 g) suspended in a 25 mL of n-hexane/isopropanol mixture (8:2) and sonicated for 1 min to give a uniform suspension. Packing was carried out at 350-500 bar with n-hexane as the solvent. After approximately eighty column volumes had passed through the column, the packing was stopped.

Chromatographic Conditions

The columns under investigation (250 mm x 4.6 mm ID) are all prepared from *tris*(3,5-dimethylphenylcarbamoyl) cellulose (CS-1, Figure 2) bound onto a 7 μm aminopropylsilanized silica gel with a pore size of 100 nm (Nucleosil 1000-7). The theoretical plate number of columns was determined for benzene using n-hexane/isopropanol (9:1) as the eluent at a flow rate of 0.5 mL/min (see Table 1). The dead volume (t_0) of the columns was measured with 1,3,5-tri-*tert*-butylbenzene, known as a nonretained compound. The mobile phase in all cases was 10% of isopropanol and 90% of n-hexane with flow rate of 0.5 mL/min for analytes 1-4 and 6, and 1 mL/min for the others.

RESULTS AND DISCUSSION

The two principal coating methods for the preparation of CSPs based on *tris*(3,5-dimethylphenylcarbamoyl) cellulose have been described in the literature. Zhang and Francotte[20] reported the use of the precipitation method. In particular, the cellulose derivative was dissolved in tetrahydrofuran and silica gel (particle size 10 μm, pore size 400 nm), previously modified with 3-aminopropyltriethoxysilane, to give 25% w/w loading (weight percent cellulose derivative to silica gel) was suspended in the solution. During moderate magnetic stirring, heptane (usually 5 to 6 times the volume of solvent used) was added dropwise to the above suspension until the cellulose derivative completely precipitated on the surface of the silica. The evaporation method has been described by Okamoto and coworkers in several papers.

In one of the most recent articles[15] silica gel (particle size 7 μm; pore size 100 nm) previously treated with 3-aminopropyltriethoxysilane, was added to a solution of the cellulose derivative in acetone to give 25% w/w loading, and dispersed as uniformly as possible. Then the solvent was evaporated under reduced pressure. This method has been applied by using different types of silica as well as organic solvents.

With these two types of CSPs, HPLC columns were packed by a slurry method and tested for a range of racemates. In both kinds of approaches neither specific advantages nor elucidation of the reasons for selecting one method instead of the other is disclosed. Moreover, although several racemates tested are identical, there is no ground for a reliable comparison of the reported chromatographic results since the experimental conditions are extremely different.

In the present work, the comparison between the two coating methods has been performed by fixing the characteristic of the silica gel (particle size 7 μm and pore size 100 nm), the loading (25% w/w), the organic solvent (acetone), and the column packing conditions. In such a way, by also fixing the chromatographic conditions, only the effect of the coating method is expected to influence the column performance. Moreover, in the range of racemates tested, several compounds already used have been included.

The CSPs obtained by either of the two methods were packed in the HPLC columns before and after sieving through a 28 μm sieve. Four different columns were prepared in this way and tested in enantioseparation.

The results of enantioseparation of racemates **1-10** on the stationary phases A-unsieved and A-sieved, prepared by coating of the silica with CS-1 by evaporation, are given in Table 2. As shown in Table 3 are the results

Table 2

Parameters Obtained for Enantioselective Chromatography on A-Unsieved and A-Sieved Columns with Racemic Analytes 1-10

Analyte	k_2' A-unsieved/ A-sieved	α A-unsieved/ A-sieved	R_s A-unsieved/ A-sieved
1	2.23/2.15	2.19/2.18	3.40/5.76
2	2.70/2.71	1.37/1/37	2.00/3.33
3	1.77/1.79	1.31/1.32	1.19/2.05
4	2.10/2.13	1.78/1.81	2.68/4.97
5	5.07/5.41	1.52/1.47	2.21/4.50
6	2.37/2.26	1.25/1.28	0.80/1.18
7	2.10/2.11	1.15/1.15	0.62/1.15
8	5.90/5.37	1.51/1.75	1.94/2.94
9	11.43/9.78	1.47/1.52	2.22/4.12
10	1.70/1.74	1.20/1.24	<0.5/1.47

Table 3

Parameters Obtained for Enantioselective Chromatography on B-Unsieved and B-Sieved Columns with Racemic Analytes 1-10

Analyte	k_2' B-unsieved/ B-sieved	α B-unsieved/ B-sieved	R_s B-unsieved/ B-sieved
1	2.08/2.10	2.08/2.21	5.65/6.38
2	2.62/2.60	1.44/1.48	3.45/3.90
3	1.70/1.71	1.23/1.33	1.48/2.04
4	1.93/2.02	1.79/1.84	4.43/5.05
5	4.90/4.97	1.55/1.60	3.78/4.80
6	2.42/2.24	1.23/1.35	1.15/1.24
7	1.97/5.50	1.16/1.16	0.84/1.16
8	5.43/5.50	1.54/1.73	2.48/3.47
9	9.83/9.82	1.44/1.55	2.73/4.45
10	1.83/1.89	1.31/1.32	1.73/1.48

Table 4

Parameters Obtained for Enantioselective Chromatography on Commercial Chiralcel OD Column with Racemic Analytes 1-10

Analyte	k_2'	α	R_s
1	1.75	2.13	8.12
2	1.97	1.43	5.38
3	1.32	1.22	1.98
4	1.49	1.84	7.08
5	3.20	1.55	6.30
6	2.07	1.29	1.47
7	2.05	1.15	1.16
8	5.50	1.56	3.40
9	9.20	1.50	4.35
10	1.17	1.30	1.82

obtained on stationary phases B-unsieved and B-sieved prepared by depositing of chiral selector onto silica by precipitation. For comparison, the enantioseparation results obtained on commercial *Chiralcel OD* column are shown in the Table 4.

Tris(3,5-dimethylphenylcarbamoyl) cellulose possess high resolving power for the compounds bearing a carbonyl, hydroxyl, or ether oxygen that can form hydrogen bonds to the N-H groups of the carbamate moiety. Two methyl groups attached on phenyl having electron-donor character and this selector effectively resolves polar compounds such as alcohols, epoxides, etc. (analytes 1, 2, 4, 5, 8, 9) and somewhat less effectively for the other analytes.

Remarkable differences in the chromatographic quality of materials obtained by different methods before sieving was observed. Column A-unsieved gave a much broader peak than B-unsieved column, for illustration see Figure 3, indicating that material obtained by an evaporation method contain considerable amount of agglomerated particles. Separation factors (α values) are similar for both materials, though resolution factors (R_S) are much higher because of the sharper peaks for the column packed with material obtained by precipitation method. Interestingly, α-values for B-sieved material are higher even those determined for Daicel *Chiralcel OD* column, cf. Tables 3 and 4.

Comparison of the results obtained with unsieved and sieved material shows positive effects of sieving of stationary phases in both cases (Figure 4). Because of the large part of agglomerated particles, this effect is even stronger

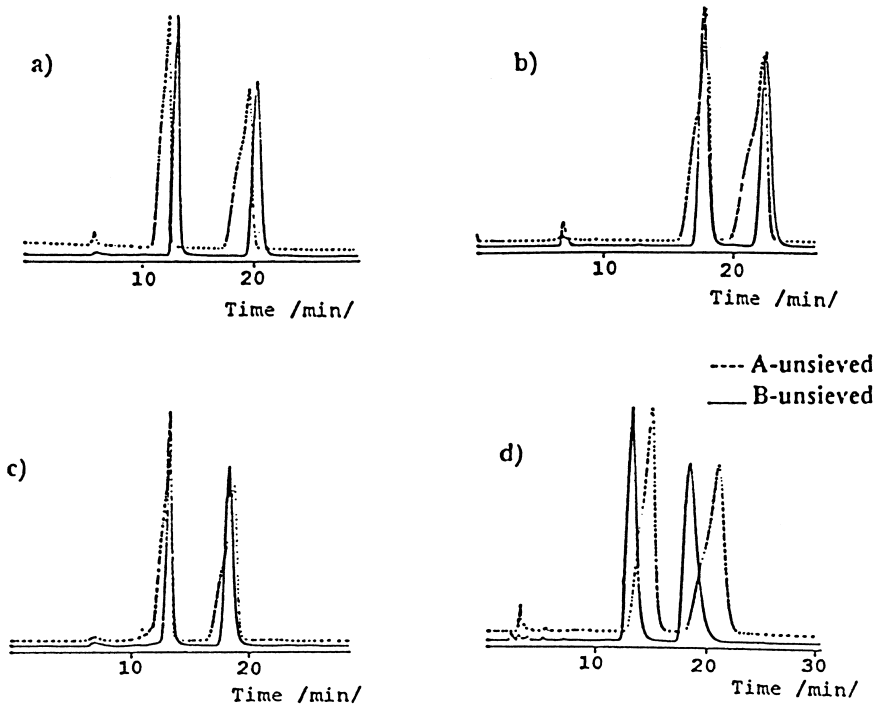

Figure 3. The comparison of chromatograms obtained by A- and B-unsieved HPLC columns for a) *trans*-stilbene oxide (1); b) flavanon (2); c) benzoin methyl ether (4); d) phenyl ethyl amine phenyl amide (8).

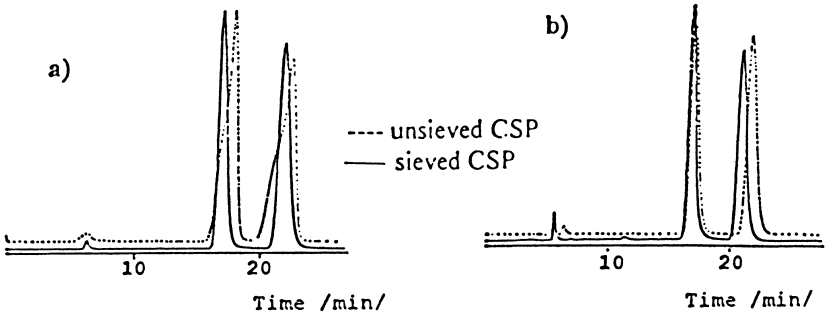

Figure 4. The comparison of chromatograms obtained for flavanon before and after sieving on a) A-sieved and A-unsieved columns; b) B-sieved and B-unsieved columns.

Table 5

Comparison of α an R_s Values for the *tris*(3,5-Dimethylphenylcarbamoyl)-Cellulose Based Columns of Various Origins

	α				R_s		
Analyte	α^a	α^b	α^c	α^d	R_s^a	R_s^c	R_s^d
1	1.68	2.36	2.21	2.13	3.22	6.38	8.12
2	1.41	---	1.48	1.43	3.08	3.90	5.38
3	1.32	1.18	1.33	1.22	1.92	2.04	1.98
5	1.58	1.59	1.60	1.55	4.38	4.80	6.30

[a] From the refs. 9,15. [b] From the ref. 20. [c] This paper; column **B-sieved**.
[d] Commerical *Chiralcel OD* column.

for material obtained by evaporation method. After sieving the deference between two stationary phases was not so large; the column filled with material obtained by precipitation showed only slightly better chromatographic and enantioseparation characteristics. Loss of material on sieving is remarkably higher for products obtained by evaporation method (46%) than products obtained by precipitation method (13%).

Comparison of the results presented here, with corresponding literature data, (Table 5) revealed, in average, higher chiral discrimination (α) of the columns B-sieved than those reported in the literature.[9,15,10] In comparison with commercially available *Chiralcel OD* columns the columns of B-sieved type exhibit approximately the same discrimination capacity and somewhat lower R_S values. The latter factor of B-sieved columns can be improved by ulterior enhancement of the size uniformity of the CSPs particles, e.g. by the sieving through the smaller-size sieve.

CONCLUSION

In conclusion we can state that stationary phase obtained by precipitation of *tris*(3,5-dimethylphenylcarbamoyl) cellulose on silica gel, and not additionally sieved, has better chromatographic and enantioselection characteristics than similar material obtained by evaporation method. After sieving through the 28 μm sieves this difference is not so straightforward, but the columns prepared from material obtained by precipitation method (B-sieved) still exhibit reproducibly better chiral separation characteristics. The loss of material on sieving is remarkably higher for products obtained by evaporation

method (46%) than for products obtained by precipitation method (13%). Comparison of the results with separation parameters from the literature, and those for commercially available columns with the same CSP revealed that the method that combines precipitation and sieving produces the highest quality CSP.

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