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Effects of β-cyclodextrin in the mobile phase on the retention and indirect detection of non-electrolytes in reversed-phase liquid chromatography

II. Steroids

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ABSTRACT

    The effect of β-cyclodextrin on the chromatographic retention time of two steroids, pregnanolone and progesterone is described. The apparent formation constant of the inclusion complexes has been calculated, and the optimization of indirect photodetection of pregnanolone, in reversed-phase chromatography, has been investigated. The presence of β-cyclodextrin in the mobile phase appears essential to enhance the detection of pregnanolone.

    Over the past few years, a great number of papers have been published on the separation, detection and quantitation of steroids. These techniques can be divided into two classes: (a) specific methods, such as colorimetry, fluorimetry, isotopic techniques and immunological assays; these are employed mainly for the quantitation of steroids in physiological fluids [1,2]. (b) Separation techniques that permit quantitation of steroids by UV photometric, fluorimetric or electrochemical detection following high-performance liquid chromatography (HPLC), or flame ionization or mass spectrometric detection following gas chromatography (GC) [3–5].
    For the quantitation of steroids in pharmaceutical preparations, two techniques are mainly employed: HPLC with UV detection and GC with flame ionization detection. In the case of pregnanolone, detection is difficult because this steroid has poor UV absorption. Thus we decided to develop a simple method using indirect photodetection following HPLC, and avoiding any derivatization. In this technique, a UV-absorbing probe is included in the mobile phase and distributed on a stationary phase. The injection of non-absorbing species disturbs the equilibrium. This gives a peak at the retention time of the non-UV-absorbing species, followed or preceded by a system peak.

Indirect photodetection has previously been applied to the detection of non-UV-absorbing ionic and non-ionic compounds [6–12]. Furthermore, the inclusion complexation of steroids with β-cyclodextrins (βCD) has been described by many authors [13–16]. Generally, the steroids form complexes with βCD in a 1:1 or 1:2 ratio.

In our previous paper [17], we described the role of βCD in the chromatographic requirements and the indirect photodetection of alcohols. This paper deals with the role of βCD in the chromatographic behaviour of pregnanolone and progesterone and the indirect photodetection of these steroids.

EXPERIMENTAL

Apparatus

The chromatographic equipment included a pump (model 110B, Beckman Instruments, France), a filter wavelength detector (Model 160, Beckman Instruments) with a 10-mm flow-cell length, a 20-μl loop injector (Altex 210A valve, USA) and an integrator (Model CR5A, Shimadzu, Japan). The columns and guard columns (100 × 4.6 mm I.D. and 1.5 × 4.6 mm I.D., respectively) were made of stainless steel equipped with swagelock connectors.

Chemicals and reagents

Methanol of HPLC grade was purchased from Prolabo (France). Pregnanolone, progesterone and β-cyclodextrin were from Sigma (France).

Chromatography

Separations were carried out with columns packed by Interchim (France) with Nucleosil C$_4$, 5 μm particle size, porosity 300 Å (Macherey-Nagel, Germany); RP2, 10 μm particle size, porosity 120 Å (Applied Biosystems, USA).

The room temperature was 20°C ± 1°C, and the air was conditioned. The flow-rate was 0.8 ml/min. The samples were injected as solutions in the mobile phase. Peak areas were determined with an integrator after conversion of results in square centimetres by planimetry. The void volume was obtained from the front peak of the chromatogram.

RESULTS AND DISCUSSION

Influence of βCD on capacity factor of steroids

The addition of βCD in the eluent decreases the retention time of the injected steroids. Figs. 1 and 2 show, for pregnanolone and progesterone respectively, the variation of the capacity factor with increasing concentrations of βCD in the eluent. The retention times decreased as the concentration of βCD increased. This reveals the formation of inclusion complexes between steroids and βCD. Moreover, this dependence varies with the methanol content of the eluent.

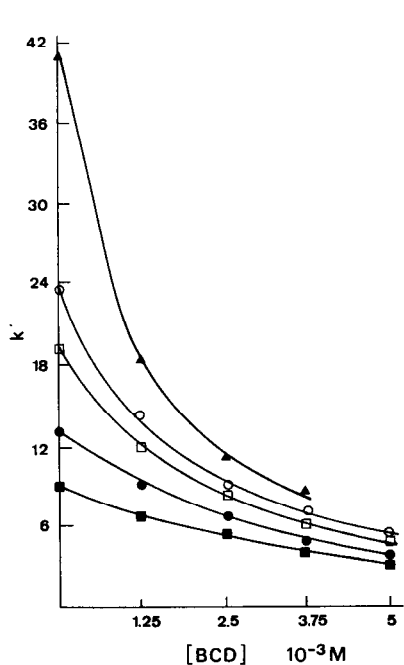Using the method previously introduced by Love and Aranhyanart [18] and

Fig. 1. Variation of pregnanolone capacity factor with increasing concentration of βCD at different proportions of methanol in the mobile phase: (▲) [CH₃OH] = 45%; (○) [CH₃OH] = 47%; (□) [CH₃OH] = 50%; (●) [CH₃OH] = 53%; (■) [CH₃OH] = 55%. Experimental conditions: column (10 cm × 4.6 mm I.D.), particle size 5 μm, 300 Å; guard column (1.5 cm × 4.6 mm I.D.), C₄; particle size 5 μm, 300 Å; flow-rate, 0.8 ml/min; detection wavelength, 280 nm; sample injected, 10 μg.
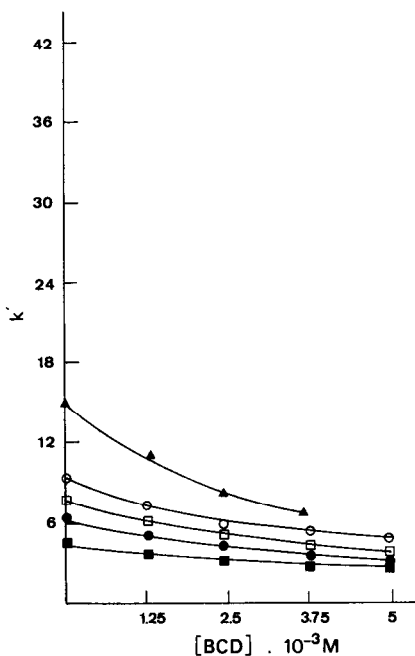
Fig. 2. Variation of progesterone capacity factor with increasing concentration of βCD at different proportions of methanol in the mobile phase: (▲) [CH₃OH] = 45%; (○) [CH₃OH] = 47%; (□) [CH₃OH] = 50%; (●) [CH₃OH] = 53%; (■) [CH₃OH] = 55%. Experimental conditions as in Fig. 1 except detection wavelength, 254 nm.

Fujimura et al. [19], we have determined the apparent stability constant of the complexes from the following equation:

$$\frac{1}{k'} = \frac{1}{k'_0} + \frac{[CD]_T}{K_D k'_0}$$

where k' is the capacity factor in the presence of βCD, K_D the dissociation constant of the complex, k'_0 the capacity factor without βCD and $[CD]_T$ the total concentration of βCD in mobile phase.

Table I lists the results for different percentages of methanol. These show that the steroid–βCD inclusion complexes have a higher stability when the content of methanol is low. Those results confirm the hydrophobic character of the interaction between steroids and βCD. Furthermore, the βCD–pregnanolone complex is more stable than the βCD–progesterone complex. This was suggested from the higher hydrophobic character of pregnanolone, which was revealed by its higher retention on the reversed-phase column with βCD in the mobile phase.

TABLE I

APPARENT ASSOCIATION CONSTANTS (K_f) OF INCLUSION COMPLEXES βCD–STEROIDS
FOR VARIOUS PERCENTAGES OF METHANOL IN THE MOBILE PHASE

Proportion of methanol (%, v/v)	Pregnanolone K_f (M^{-1})	Progesterone K_f (M^{-1})
45	953	347
47	670	240
50	635	261
53	540	216
55	345	165

Our results can be compared with those from a previous determination of the
stability of the progesterone–βCD complex in ethanol–water (15:85, v/v) by Kralova
and Mitterhauszerova [20]. In this case, the constant calculated from solubility exper-
iments was 700 M^{-1}. By the HPLC technique, with methanol–water (45:55, v/v) as
the mobile phase, we found a value of 347 M^{-1}.

Indirect detection

As progesterone has a UV molar absorptivity ($\varepsilon_{254} = 12\ 700$) much greater
than that of pregnanolone ($\varepsilon_{max} = \varepsilon_{280} = 150$) we used the first steroid as a marker in
the eluent in order to detect the injected second one.

Fig. 3 shows an example of chromatographic profile obtained by using a mobile
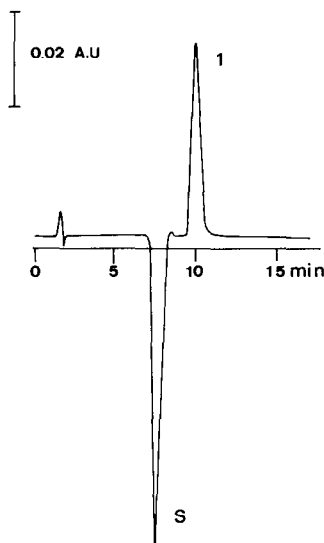

Fig. 3. Chromatographic profile of pregnanolone with progesterone as probe. Mobile phase, methanol–
water (47:53, v/v); progesterone concentration, 0.032 mM; βCD concentration 4.9 mM. Peaks: S = system
peak; 1 = pregnanolone. Experimental conditions as in Fig. 1, except detection wavelength, 254 nm.

phase containing both βCD (4.9 mM) and progesterone (0.032 mM) and injecting pregnanolone. The first signal (negative) corresponds to the retention time of progesterone obtained in the same eluent without progesterone. The second signal (positive) corresponds to the retention time of pregnanolone in the latter eluent.

We plotted the positive peak area as a function of the injected amount of pregnanolone. Several experiments corresponding to different concentrations of βCD were performed, and gave the calibration curves shown in Fig. 4. It appears that the slope of the calibration lines increases as the βCD concentration increases.
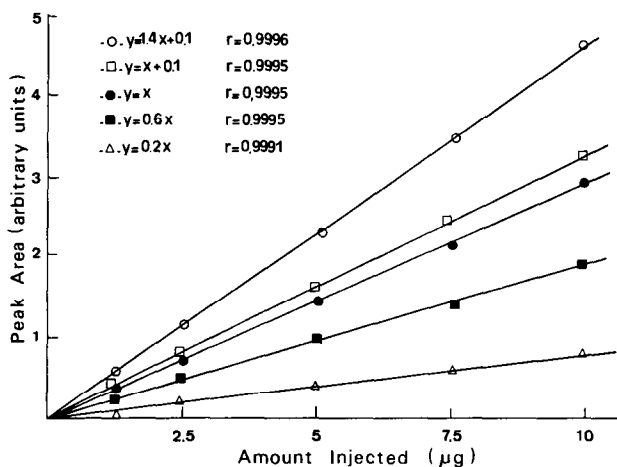

Fig. 4. Calibration curves of pregnanolone at various concentrations of βCD in methanol–water (47:53, v/v) mobile phase with progesterone (0.032 mM) as probe. (△) [BCD] = 0 mM; (■) [BCD] = 1.23 mM; (●) [BCD] = 2.45 mM; (□) [BCD] = 3.7 mM; (○) [BCD] = 4.9 mM. Experimental conditions as in Fig. 1, except detection wavelength, 254 nm (unless measured at λ_{max} = 280 nm). $y = Ax + B$, where A = slope and B = intercept; r = regression coefficient.

The peak area corresponding to the injection of a constant amount of pregnanolone (10 μg) was measured in the presence of various amounts of βCD in the eluent. The results are shown in Fig. 5. The concentration of βCD is limited by its solubility in the eluent: under the present experimental conditions (methanol–water 47:53 v/v), its maximum solubility is $ca.$ 5 mM.

We also studied the influence of methanol content in mobile phase at a constant βCD concentration of 4.9 mM. Fig. 6 shows the results obtained: the slope of calibration curves is greatest for the lowest concentration of methanol. Owing to the limited solubility of progesterone in the eluent, a 47% concentration of methanol is the minimum value for a complete solubilization of this compound.

As previously stated, the indirect detection of a solute gives a maximum response when the capacity factors of the solute and the probe are similar (6–12). In this case, increasing concentrations of βCD in the eluent decrease the separation factor α, defined as the ratio of the capacity factors of pregnanolone and progesterone. The same effect is observed as the methanol concentration in the eluent is decreased. These two effects contribute to an enhancement of pregnanolone detection.
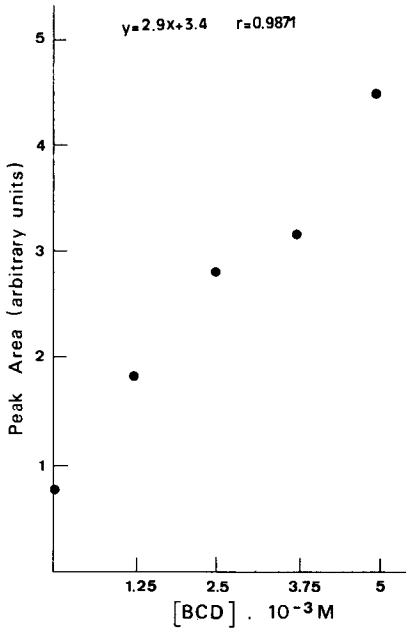
Fig. 5. Peak area of pregnanolone (10 μg) plotted *versus* the concentrations of βCD in methanol–water (47:53, v/v) mobile phase with progesterone (0.032 m*M*) as probe. Experimental conditions as in Fig. 4.

For example, α varies from 1.47 to 1.37, these values corresponding respectively to 3.7 and 4.9 m*M* βCD in the mobile phase methanol–water (53:47, v/v). With 3.7 m*M* βCD, α varies from 1.36 to 1.47 when the proportion of methanol is increased from 47% to 53% (v/v).
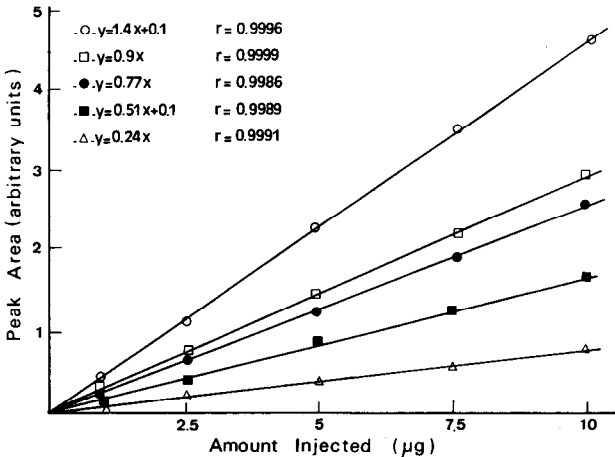

Fig. 6. Calibration curves of pregnanolone at various concentrations of methanol in the mobile phase containing 4.9 m*M* of βCD: (△) see Fig. 4; (○) [CH₃OH] = 47%; (□) [CH₃OH] = 50%; (●) [CH₃OH] = 53%; (■) [CH₃OH] = 55%. Experimental conditions as in Fig. 4.

   In order to verify this property, we studied the variation of the apparent molar absorptivity as a function of α. The apparent molar absorptivity, ε^*, was defined by Hackzell and Schill [6] with the following equation:

$$\varepsilon^* = \frac{ysu}{mdb}$$

where y is the sample peak area, s is the detector setting range, u is the flow-rate, m is the amount of compound injected, d is the chart speed and b is the path length of the detector cell. Fig. 7 shows a plot of log ε^* against log α. The detection response is at a maximum when α is close to the unity.

   The eluent composition must be chosen so that the chromatographic resolution of the two steroids is complete, in order to obtain reliable quantitative measurements. Thus, a minimum value of ($\alpha - 1$) is necessary for indirect detection measurement.

   The maximum absorption in the UV spectrum of pregnanolone is at 280 nm in ethanolic solution. We measured the value of ε^{**} at this wavelength using an eluent without a probe (progesterone), and found a value of $\varepsilon^{**} = 150$. For comparison, at 254 nm the ε^{**} value of pregnanolone is 47.

   Using indirect detection in the conditions described above, and at a wavelength of 254 nm, the ε^* value can reach 915, that is to say a six-fold enhancement for $\varepsilon^*_{254}/\varepsilon^{**}_{280}$ and a 20-fold enhancement for $\varepsilon^*_{254}/\varepsilon^{**}_{254}$.

   In contrast to other cases of indirect detection of uncharged compounds, in which the system peak appears first as a negative peak, and the solute peak emerges later as a negative peak [7,9,11], we observed a negative system peak followed by a positive sample peak on the C_4 column. This suggests that the injected pregnanolone displaces the progesterone complexed by the βCD in the mobile phase owing to the higher apparent association constant of pregnanolone–βCD mentioned above. So the
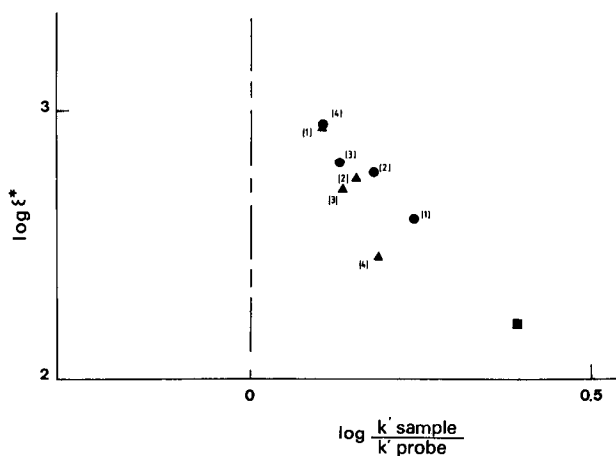

Fig. 7. Influence of the steroid capacity factor ratio on the detection sensitivity: (■) [CH$_3$OH] = 47%; (●) [CH$_3$OH] = 47% at various concentrations of βCD in the mobile phase: (1) 1.23 mM; (2) 2.45 mM; (3) 3.7 mM; (4) 4.9 mM. (▲) 4.9 mM βCD at various proportions of methanol in the mobile phase: (1) 47%; (2) 50%; (3) 53%; (4) 55%. Experimental conditions as in Fig. 4.

deficit of βCD–progesterone appears as a negative signal, and the positive peak is due to the release of pregnanolone and progesterone.

We compared these results with those obtained in the absence of βCD by selecting a mobile phase composition that gave on α value close to unity. Fig. 8 shows the chromatogram pattern obtained by injecting pregnanolone into the C_4 column and eluted by a mobile phase of methanol–water (72:28, v/v), containing 0.032 mM progesterone. This yielded an α value of 1.5, similar to the best conditions of indirect detection. As in the preceding chromatogram, there is a negative peak at the retention time of progesterone followed by a positive peak corresponding to pregnanolone.

Calculation of the molar apparent absorptivity gives $\varepsilon^* = 47$, which is similar to the ε^{**} value of pregnanolone at 254 nm measured without progesterone in the eluent. These results are in agreement with the theoretical predictions of Golshan-Shirazi and Guiochon [21] but in this case the indirect detection signal is too weak to permit improved determination of pregnanolone.
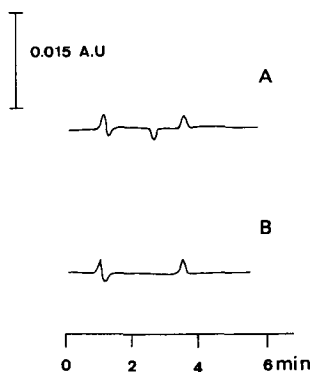

Fig. 8. Chromatographic profiles of pregnanolone without βCD in the mobile phase: (A) methanol–water (72:28, v/v) with progesterone (0.032 mM); (B) methanol–water (72:28, v/v) without progesterone. Experimental conditions as in Fig. 1. Detection wavelength: (A) 254 nm; (B) 280 nm.

Influence of the support on indirect photodetection

We compared the results obtained from the C_4 column with those obtained from a less hydrophobic support, a C_2 column, on which the capacity factors of the two steroids are very similar.

On this type of column, the value of α is 1.18 without βCD and 0.7 with βCD at 4.9 mM. This indicates an inversion of the elution order by βCD. Fig. 9 shows the chromatogram observed following injection of a pregnanolone sample eluted with βCD (4.9 mM) and progesterone (0.032 mM) in the mobile phase. A first signal (positive) appears corresponding to pregnanolone, immediately followed by a second signal (negative) corresponding to the system peak. This procedure permits the detection of pregnanolone with a ten-fold enhancement as compared with simple UV detection at 280 nm. Nevertheless, this is not sufficient (resolution 1.2) to give reliable measurements for quantitation. Several attempts to improve the separation by changing the concentrations of βCD and methanol failed. It must be concluded that the C_2
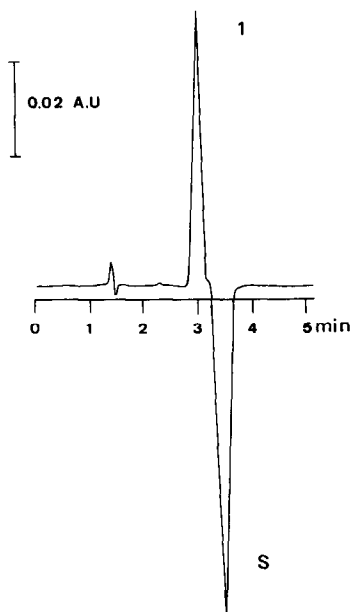
Fig. 9. Chromatographic profile of pregnanolone with progesterone as probe. Mobile phase methanol–water (50:50, v/v) containing 0.032 mM progesterone and 4.9 mM BCD. Peaks: 1 = pregnanolone; S = system peak. Column, C_2 (10 cm × 4.6 mm I.D.); particle size, 10 μm, 120 Å; flow-rate, 0.8 ml/min; detection wavelength, 254 nm; sample injection, 10 μg.

column is unable to separate the two steroid compounds with a high enough resolution to achieve a quantitative determination of pregnanolone.

CONCLUSIONS

The formation of inclusion complexes between steroids and βCD modifies their chromatographic properties on reversed-phase column eluted with methanol–water.

Because pregnanolone and progesterone have similar chromatographic properties, the latter was used as a marker in the detection of pregnanolone, which has weak UV absorbance. The detection can be enhanced by the presence of βCD in the eluent. This property, which has previously been observed in the case of alcohols [17], seems to be more general and extends the application of chromatographic indirect detection by the presence in the eluent of an additional compound, such as βCD, that is able to form inclusion complexes with the analyte and probe. Further investigation of this phenomenon is currently being undertaken.

REFERENCES

1 C. J. Munro and B. L. Lasley, *Non-Radiometric Assays: Technology and Application in Polypeptide and Steroid Hormone Detection*, Alan R. Liss, New York, 1988, p. 289.
2 H. R. Behrman, *Non-Radiometric Assays: Technology and Application in Polypeptide and Steroid Hormone Detection*, Alan R. Liss, New York, 1988, p. 1.

3 R. K. Gilpin and L. A. Pachla, *Anal. Chem.*, 61 (1989) 191.
4 K. Robards and P. Towers, *Biomed. Chromatogr.*, 4 (1990) 1.
5 H. L. J. Makin and E. Heftman, *High Performance Liquid Chromatography of Steroid Hormones*.
6 L. Hackzell and G. Schill, *Chromatographia*. 15 (1982) 437.
7 P. Herné, M. Renson and J. Crommen, *Chromatographia*, 19 (1984) 274.
8 L. Hackzell, T. Rydberg and G. Schill, *J. Chromatogr.*, 282 (1983) 179.
9 J. E. Parkin, *J. Chromatogr.*, 287 (1984) 457.
10 J. Crommen and P. Herné, *J. Pharm. Biomed. Anal.*, 2 (1984) 241.
11 J. E. Parkin and H. T. Lau, *J. Chromatogr.*, 314 (1984) 48.
12 J. Crommen, G. Schill, D. Westerlund and L. Hackzell. *Chromatographia*, 24 (1987) 252.
13 S. G. Frank and D. R. Kavaliunas, *J. Pharm. Sci.*, 72 (1983) 1215.
14 D. W. Armstrong, A. Alak, K. Bui, W. Demond, T. Ward, T. E. Riehl and W. L. Hinze, *J. Inclusion Phenomena*, 2 (1984) 533.
15 M. Gazdag, G. Szepesi and L. Huszar, *J. Chromatogr.*, 351 (1986) 128.
16 A. Bethod, H. L. Jin, T. E. Beesley, J. D. Duncan and D. W. Armstrong, *J. Pharm. Biomed. Anal.*, 8 (1990) 123.
17 M. Gosselet and B. Sebille, *J. Chromatogr.*, 552 (1991) 563–573.
18 L. J. Cline Love and M. Arunhyanart, *ACS Symp.*, 297 (1986) 226.
19 K. Fujimura, T. Ueda, M. Kitagawa, H. Takayanagi and T. Ando, *Anal. Chem.*, 58 (1986) 2668.
20 K. Kralova and L. Mitterhauszerova, *Pharmazie*, 44 (1989) 623.
21 S. G. Shirazi and G. Guiochon, *Anal. Chem.*, 62 (1990) 923.