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Indirect photodetection of pregnanolone on a Cyclobond column by high-performance liquid chromatography

Benoit Agnus[a], Noëlle-Martine Gosselet*[,b], Bernard Sebille[b]

[a]*Laboratoires Besins Iscovesco, 10 Rue Morel, 92120 Montrouge, France*

[b]*Laboratoire de Physico-Chimie des Biopolymères, CNRS–Université Paris XII, UM 27, 2 Rue Henri Dunant, 94320 Thiais, France*

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Abstract

The chromatographic properties of eight steroids were studied on a Cyclobond column in order to select those which can be used as markers for the indirect photodetection of pregnanolone. Testosterone was shown to be the best compound, enhancing the pregnanolone detection by a factor of 6.5 with respect to the direct detection of pregnanolone at 280 nm under the optimum conditions: 0.0085 m*M* testosterone with methanol–water (65:35) as mobile phase. The influence of the percentage of methanol and the marker concentration in the mobile phase was studied. The models developed previously on a reversed-phase column were successfully applied and the apparent inclusion constants for progesterone and testosterone in the β-cyclodextrin cavity were determined. They were 103 and 2200 l mol^{-1} for progesterone and testosterone, respectively, with methanol–water (65:35) and 50 and 780 l mol^{-1} with methanol–water (75:35) as mobile phase.

1. Introduction

Cyclodextrins are natural macrocyclic polymers of glucose in toroidal shape. They have the ability to trap molecules in their hydrophobic internal cavity, thus forming inclusion complexes.

Immobilized cyclodextrin stationary phases developed by Armstrong and Demond [1,2] were essentially used for the separation of enantiomers [2–6], diastereoisomers [6], and structural isomers [7–9]. However, a cyclodextrin bonded phase column behaves also as a conventional phase when it is used with water–organic mixtures as mobile phase for the separation of barbiturates [2] and vitamins [8].

Compounds with a poor detector response can be detected and determined using indirect ultraviolet or fluorescence detection. In this technique an ultraviolet- or fluorescence-absorbing species is added to the mobile phase. The detection results from perturbation of the distribution equilibria of the probe on injection of the sample. Reversed-phase chromatography is the main field of application of the indirect detection of both charged and uncharged species. In this work, we examined the possibility that pregnanolone may be detected by indirect photodetection on a β-cyclodextrin bonded phase (Cyclobond I) for comparison with results obtained previously on a C_4 reversed-phase column

* Corresponding author.

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in the presence of β-cyclodextrin in the mobile phase [10]. Pregnanolone has a low molar absorption coefficient, $\varepsilon = 500$ l mol^{-1} cm^{-1}, at 280 nm and its more sensitive detection is of interest.

2. Experimental

2.1. Apparatus

The chromatographic equipment included a pump (Varian Model 5500), a variable-wavelength detector (Varian Model UV 200) with a 9-mm flow cell length, a 20-μl loop injector (Rheodyne Model 7125), an integrator for data acquisition (Spectra-Physics Model 4400) and a diode-array detector (Varian UV 9065). The columns (250 × 4.6 mm I.D.) were made of stainless steel equipped with Swagelock connectors.

2.2. Chemicals and reagents

Methanol of HPLC grade was purchased from SDS (France). Progesterone, testosterone, estradiol and pregnanolone were purchased from Diosynth (Netherlands) and the other steroids from Sigma (France).

2.3. Chromatography

Separations were carried out with a Cyclobond I column (250 × 4.6 mm I.D.) purchased from Astec (USA) and used at room temperature (20 ± 1°C). The flow rate was 0.6 ml min^{-1}. The samples were injected as solutions in the mobile phase. Peak areas were determined with the integrator and were expressed in arbitrary peak-area units. The void volume was obtained from the methanol front peak of the chromatogram.

3. Results and discussion

3.1. Choice of the probe

In order to select the best probe for the indirect photodetection of pregnanolone, which has a low absorption at 280 nm, we studied the chromatographic properties of eight steroids (see Table 1). The capacity factors (k') reflect the different stabilities of the inclusion complexes, the highest corresponding to testosterone. As expected, all the k' values decrease with increasing amount of methanol in the mobile phase.

The selectivity factor, α_s, of each steroid calculated with respect to the steroid to be detected, pregnanolone, is also reported in Table 1. Depending on the stability of the inclusion complexes and the steric hindrance of the steroid, the variation of α_s with methanol concentration in the mobile phase is large or small. The α_s value calculated for testosterone with respect to pregnanolone does not vary with increasing concentration methanol. The α_s value calculated for progesterone decreases with increasing concentration methanol, in contrast to the α_s values for the other steroids, which increase with increasing methanol content.

Owing to their different selectivities, which are greater or smaller than 1, and their high molar absorption coefficients, progesterone and testosterone were selected to serve as markers for the indirect detection of pregnanolone. The molar absorption coefficients are $\varepsilon = 13\,700$ and 12 000 l mol^{-1} cm^{-1}, respectively.

Table 1
Capacity factors and selectivity factors for the studied steroids on a Cyclobond I column

Compound	Methanol–water					
	85:15		75:25		65:35	
	k'	α_s	k'	α_s	k'	α_s
Pregnanolone	0.52	–	1.08	–	2.60	–
Progesterone	0.38	1.37	0.64	1.69	1.46	1.78
Testosterone (T)	1.22	0.43	2.34	0.46	5.90	0.32
T. propionate	0.60	0.87	1.34	0.81	3.96	0.66
T. benzoate	1.02	0.51	2.52	0.43	8.22	0.32
Estradiol (E)	0.28	1.86	0.70	1.54	2.44	1.27
E. propionate	0.50	1.04	1.32	0.82	4.30	0.61
E. benzoate	0.38	1.37	1.10	0.98	3.70	0.70

3.2. Qualitative results

The chromatograms in Figs. 1 and 2 show the possibility of indirect photodetection of pregnanolone with progesterone (Fig. 1a) and testosterone (Fig. 2a) as markers with methanol–water (85:15, v/v) as mobile phase. For each case, we compared the response of indirect photodetection with that of direct photodetection of pregnanolone at 280 nm. For 0.048 m*M* progesterone as marker in the mobile phase, there was a 1.4-fold enhancement of the pregnanolone signal. For 0.017 m*M* testosterone as enhancement by a factor 1.1 was observed.

The direction of the marker and sample peaks differs according to the marker selected. We observed, as previously reported by Schill and Crommen [11] for uncharged species, a positive sample peak when k'_{sample} is lower than k'_{marker} (Fig. 2a) and a negative sample peak when k'_{sample} is greater than k'_{marker} (Fig. 1a). The peak corresponding to the marker, negative or positive, is marked out by the system peak (SP).

In order to identify the species corresponding to the different peaks, we used a diode-array detector. With progesterone as marker (Fig. 1b) the first chromatographic peak corresponds to progesterone and the second is the sum of a known amount of pregnanolone (32 nmol) with a deficiency in progesterone. When the testosterone marker is used (Fig. 2b) the first chromatographic peak, which appears at the retention time of pregnanolone, corresponds to the simultaneous presence of pregnanolone and testosterone, and the second peak, which appears at the retention time of testosterone, corresponds to a deficiency in testosterone.

3.3. Quantitative results

For the determination of the optimum conditions for the indirect detection of pregnanolone, we varied the concentrations of the marker and methanol in the mobile phase.

Figs. 3 and 4 show a linear correlation between the response and the amount of pregnanolone injected (8–64 nmol), the concentrations varying from 0.0032 to 0.048 m*M* for

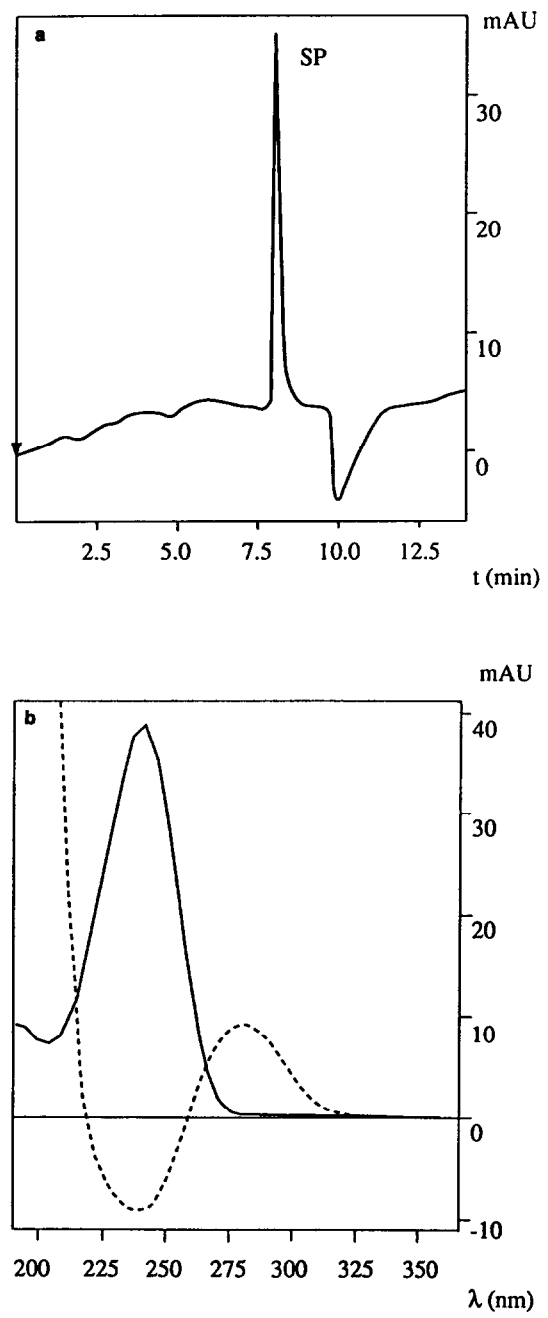
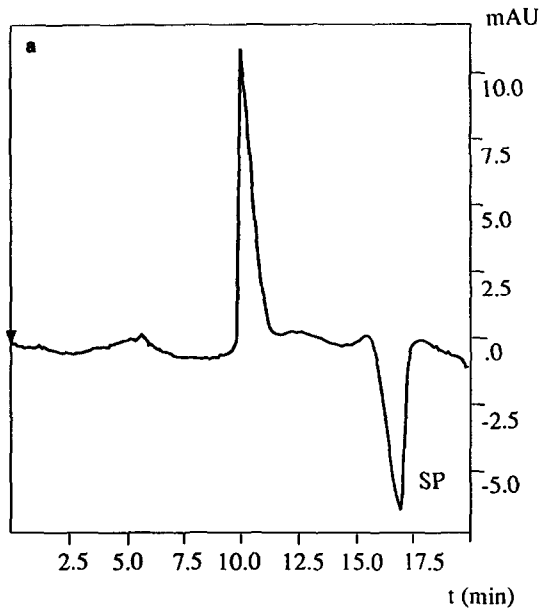


Fig. 1. (a) Indirect chromatographic detection of pregnanolone with progesterone as probe. Chromatographic conditions: mobile phase, methanol–water (85:15, v/v); λ = 241 nm; flow-rate, 0.6 ml min^{-1}; amount of pregnanolone injected, 32 nmol. SP refers to system peak, *i.e.*, progesterone. (b) Diode-array detector spectra corresponding to (solid line) first peak and (dashed line) second peak.



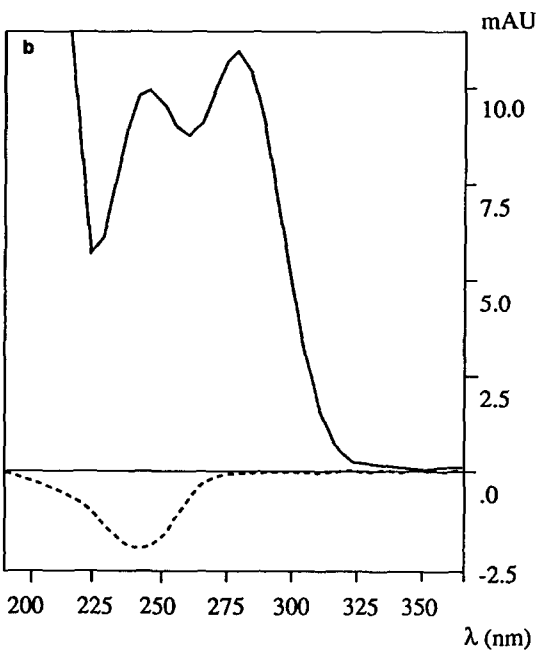


Fig. 2. (a) Indirect chromatographic detection of pregnanolone with testosterone as probe. Chromatographic conditions as in Fig. 1. SP is the system peak, *i.e.*, testosterone. (b) Diode-array detector spectra corresponding to (solid line) first peak and (dashed line) second peak.

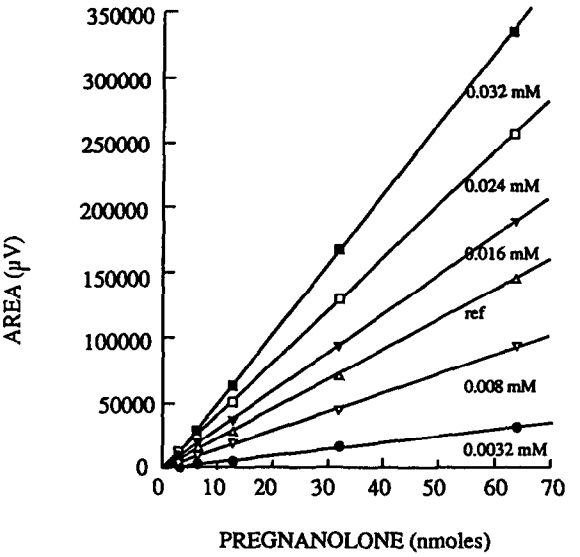


Fig. 3. Quantitative indirect detection of pregnanolone with various concentrations of progesterone with methanol–water (65:35) as mobile phase. Other chromatographic conditions: flow-rate, 0.6 ml min^{-1}; $\lambda = 241$ nm;. The reference curve (△) corresponds to direct detection of pregnanolone at 280 nm.

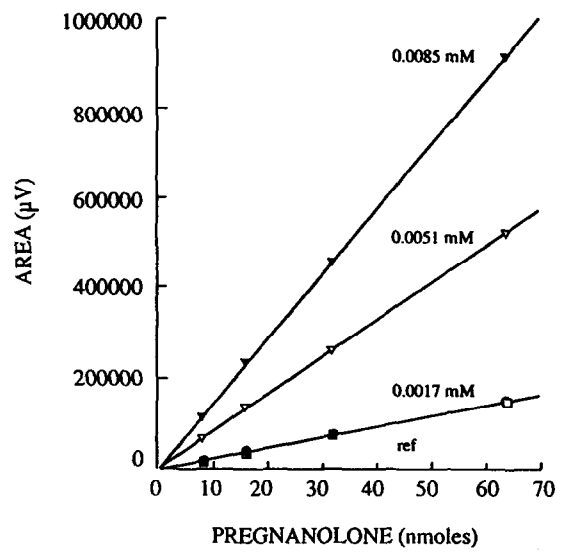


Fig. 4. Quantitative indirect detection of pregnanolone with various concentrations of testosterone with methanol–water (65:35) as mobile phase. Other chromatographic conditions: flow-rate, 0.6 ml min^{-1}; $\lambda = 241$ nm. The reference curve (●) corresponds to direct detection of pregnanolone.

progesterone or from 0.0017 to 0.0085 m*M* for testosterone when methanol–water (65:35) is used as the mobile phase. The highest response is obtained for the highest concentration in progesterone or testosterone in the mobile phase. With 0.032 m*M* progesterone and methanol–water (65:35) an enhancement of the pregnanolone signal by a factor of 2.4 is observed compared with the direct detection of pregnanolone at 280 nm. With 0.0085 m*M* testosterone and methanol–water (65:35) the signal is enhanced by a factor of 6.5.

For higher methanol concentrations in the mobile phase, we also observed linear correlations between the response and the amount of pregnanolone injected.

Figs. 5 and 6 show the influence of methanol on the response. An increasing methanol content in the eluent decreases the response. The variation of the response is not proportional to the amount of methanol.

Crommen and co-workers [11–13] developed three models to explain the sense and the intensity of the peaks observed with indirect photodetection. These models were developed

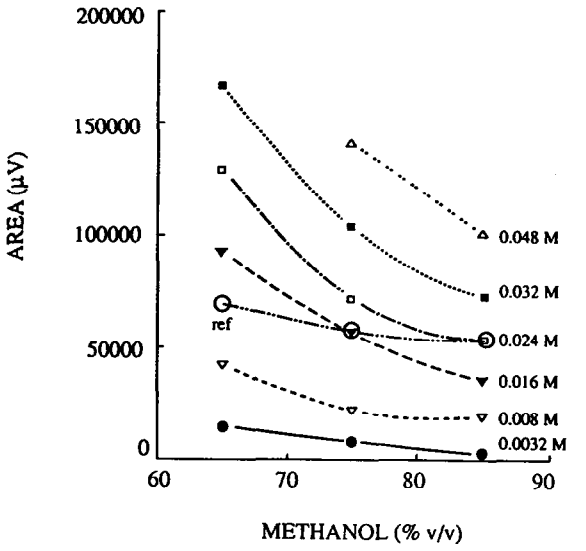


Fig. 5. Influence of methanol on the indirect response for the progesterone probe. Flow rate, 0.6 ml min^{-1}; $\lambda = 241$ nm; amount of pregnanolone injected, 32 nmol. The reference curve (○) corresponds to direct detection of pregnanolone at 280 nm.

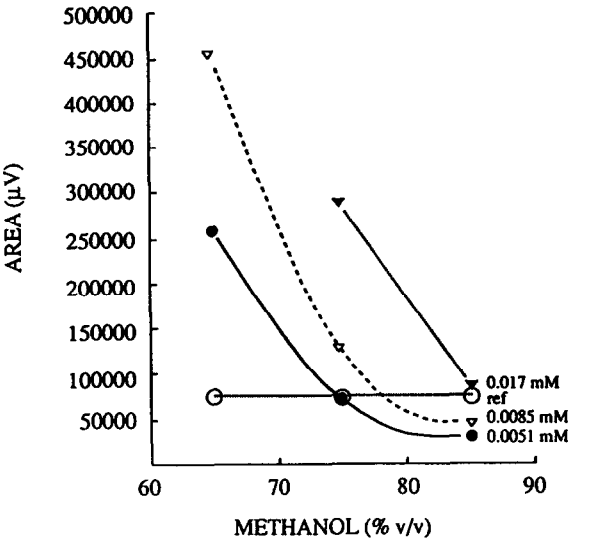


Fig. 6. Influence of methanol on the indirect response for the testosterone probe. Flow rate, 0.6 ml min^{-1}; $\lambda = 241$ nm; amount of pregnanolone injected, 32 nmol. The reference curve (○) corresponds to direct detection of pregnanolone at 280 nm.

on the assumption of a pure Langmuir adsorption isotherm on a C_{18} stationary phase, which was also assumed for the Cyclobond column. Therefore, we applied the Crommen models [12] after calculating the $(\varepsilon^*/\varepsilon)$ ratio according to Hackzell and Shill [14]:

$$\varepsilon^* = \frac{ysu}{mdb} \tag{1}$$

where y is the sample peak area, s the detector range, u the flow-rate, m the amount of compound injected, d the chart speed and b the path length of the detector cell. We determined for each marker the ε value under the same conditions as ε^*.

For a system with only two components competing for the adsorption, Crommen *et al.* [12,13] gave the following equations:

$$\frac{\Delta C_k}{\Delta C_j} = \frac{\varepsilon^*}{\varepsilon} = \theta_k \cdot \frac{\alpha_s}{1-\alpha_s} \tag{2}$$

$$\frac{\varepsilon^*}{\varepsilon} \cdot \frac{1-\alpha_s}{\alpha_s} = \frac{K_k C_{k,M}}{1 + K_k C_{k,M}} \tag{3}$$

where K_k is the absorption constant of marker k

and $C_{k,M}$ its concentration in the mobile phase, ΔC_k and ΔC_j are the concentration variations of marker k and sample j, respectively, in the elution sample zone, θ_k is the partial coverage of the stationary phase by the marker and α_s is equal to the ratio of the capacity factors of sample j and marker k:

$$\alpha_s = \frac{k'_j}{k'_k} \quad (4)$$

This first model was applied successfully to the testosterone marker (Fig. 7). This model is compatible with a positive sample peak and an increasing sensitivity when the marker concentration is higher. We were then able to determine the testosterone adsorption constant, K_k, at various methanol concentrations. These K_k values are apparent inclusion constants and correspond to the slope of the straight lines representing the variation of $(\varepsilon^*/\varepsilon)[(1-\alpha_s)/\alpha_s]$ with $C_{k,M}$. The product $K_k C_{k,M}$ can be neglected with respect to unity. We found for K_k values of 2200 and 780 l mol^{-1} for 65:35 and 75:25 methanol–water mobile phases, respectively. The slopes

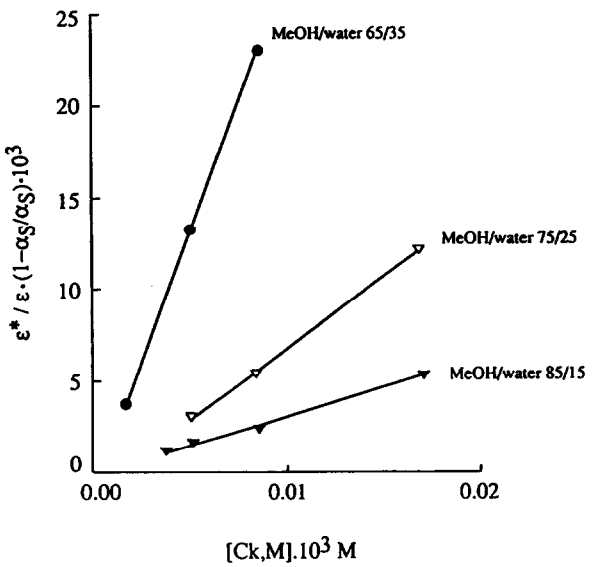


Fig. 7. Estimation of the apparent inclusion constant of testosterone from pregnanolone response. Concentration: $1.7\cdot10^{-3}$–$1.7\cdot10^{-2}$ mM in methanol–water mobile phases of the proportions indicated. Flow-rate, 0.6 ml min^{-1}; $\lambda = 241$ nm.

are positive because the peak signal is positive. These apparent constant K_k values are of the same order of magnitude as that determined by Liu *et al.* [15] for a 1:1 complex ($K_k = 5058$ l mol^{-1}) in water using phase solubility and spectroscopic techniques.

Despite the fact that the selectivity factor of progesterone is closest to unity, the highest sensitivity was obtained when testosterone was used as a marker in the mobile phase.

For a system corresponding to the simultaneous adsorption of free (j) and associated sample with the marker (jk), competing with the marker adsorption, Crommen developed a second model [12,13] taking into account the formation of the associated form (jk), according to

$$\frac{\varepsilon^*}{\varepsilon}\cdot\frac{1-\alpha_s}{\alpha_s} = \theta_k\left(1-\frac{K_{jk}}{K_k K_{j0}}\right) = K_k\left(1-\frac{K_{jk}}{K_k K_{j0}}\right)C_{k,M} \quad (5)$$

where $K_{j,k}$ is the adsorption constant of the associated sample jk, K_k that of marker k and K_{j0} that of free sample j. This equation is based on the hypothesis that the product $K_{j,k}C_{k,M}$ is much lower than K_{j0}, *i.e.*, a low fraction of sample j is associated with the marker k.

This model was used to explain the indirect detection results obtained with progesterone as marker. Fig. 8 shows a good proportionality between $(\varepsilon^*/\varepsilon)[(1-\alpha_s)/\alpha_s]$ and $C_{k,M}$. We therefore conclude that a fraction of sample j (pregnanolone) is associated with the marker k (progesterone) on the stationary phase. This association of pregnanolone and progesterone can explain the lower sensitivity of the response for the highest concentration of progesterone as compared with that obtained with testosterone, which only exists as a free species. Indeed, $(\varepsilon^*/\varepsilon)[(1-\alpha_s)/\alpha_s]$ is not equal to θ_k but to $\theta_k[1-(K_{jk}/K_k K_{j0})]$, which explains why the sensitivity does not increase as much as with testosterone. The slopes are negative, as is the direction of the signal peak. The slopes only permit an approximate K_k calculation because K_k is decreased by $1-(K_{jk}/K_{j0})$.

We found for K_k values of 103 and 50 l mol^{-1}

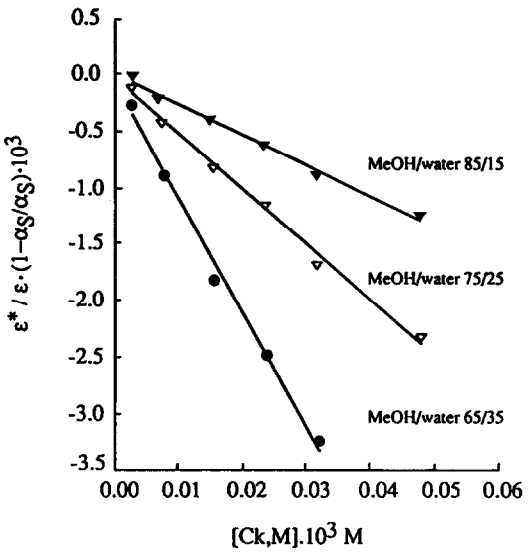


Fig. 8. Estimation of the apparent inclusion constant of progesterone from pregnanolone response. Concentration: $32 \cdot 10^{-4}$–$4.8 \cdot 10^{-2}$ m*M* in methanol–water mobile phases of the proportions indicated. Flow-rate, 0.6 ml min^{-1}; $\lambda = 241$ nm.

for 65:35 and 75:25 methanol–water mobile phases, respectively. These values are of the same magnitude as those determined previously by another method [10].

In conclusion, we have shown that indirect detection of pregnanolone was possible on a β-cyclodextrin stationary phase and that the models developed by Crommen [12,13] could be applied successfully to this particular stationary phase. Testosterone, which is the best marker, allowed us to detect 2 nmol of pregnanolone with methanol–water (65:35, v/v). These results can be compared with those obtained previously [10] on a C_4 column. We observed a sixfold enhancement of the signal obtained with indirect detection of the pregnanolone in the presence of 4.9 m*M* of β-cyclodextrin and 0.032 m*M* progesterone in comparison with the signal obtained at 280 nm by direct detection with methanol–water (47:53) as the mobile phase. The 2.4-fold enhancement observed in this work with the progesterone marker at 0.032 m*M* with methanol–water (65:35) as mobile phase, which is lower than that observed on the C_4 column, can be explained by two facts: a lower progesterone coverage on the β-cyclodextrin column than on the C_4 column, and a higher percentage of methanol in the mobile phase, which is unfavourable.

Testosterone at 0.0085 m*M* with methanol–water (65:35) as the mobile phase offers a sensitivity comparable to that obtained on a C_4 column with 0.032 m*M* progesterone with methanol–water (47:53) mobile phase. This method can be an alternative for the detection of poor UV-absorbing species, but both the sample and the marker must interact with β-cyclodextrin.

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