Journal of Chromatography, 157 (1978) 65–74

CHROM. 10,976

SEPARATION OF EPIMERS OF BUDESONIDE AND RELATED CORTICOSTEROIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

A COMPARISON BETWEEN STRAIGHT- AND REVERSED-PHASE SYSTEMS

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(First received January 5th, 1978; revised manuscript received February 24th, 1978)

SUMMARY

Liquid chromatographic conditions for epimer separations of homologous 16α,17α-acetals of 16α-hydroxyprednisolone were investigated in straight-phase systems (on μBondapak NH_2, μBondapak CN and μPorasil supports) and reversed-phase systems (on μBondapak C_{18}, μBondapak alkylphenyl and μBondapak CN supports). In the straight-phase systems the separation is practically independent of the alkyl chain length at the centre of chirality (C-22), whereas in the reversed-phase systems the separation is strongly influenced by the chain length. Long-chain ($\geq C_3$) epimers are preferentially separated by the latter systems, but straight-phase systems must be applied for the separation of short-chain epimer pairs. For separation of homologous steroids the reversed-phase systems are superior. Optimal separations of epimers were obtained using systems based on the μBondapak C_{18} and μBondapak NH_2 supports.

INTRODUCTION

A previous paper[1] has detailed the liquid chromatographic behaviour of a number of newly synthesized corticosteroids[2,3] with high anti-inflammatory potency. The investigation was carried out using a reversed-phase system based on the μ-Bondapak C_{18} packing material. The compounds studied were 16α,17α-acetals of 16α-hydroxyprednisolone with various alkyl chain lengths at C-22. The influence of fluorine substitution at the 6α and 9α positions and the esterification of the 21-hydroxyl group on retention were investigated.

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The acetalization of the 16α- and 17α-hydroxyl groups in the steroids studied leads to the introduction of a new chiral centre (C-22) and thus to the formation of two epimers[2,3]. The work aimed at evaluating the optimal chromatographic conditions for the separation of these epimers in order to facilitate quantitative determinations. It was shown that the choice of organic modifier in water was critical for the separations[1]. It was also found[1] that the reversed-phase system is highly selective with respect to the length of the hydrocarbon chain at C-22. The separation factor for epimers increased linearly from 1.0 for methyl to *ca.* 1.6 for heptyl substitution at the chiral centre. This means that a reversed-phase system is not sufficient for the complete analytical separation of all the epimers of interest.

The present work deals with different modes of high-performance liquid chromatography (HPLC), involving three reversed-phase and three straight-phase systems. As solute–stationary phase as well as solute–mobile phase interactions differ in different modes of chromatography, the use of various systems might give complementary results as shown, *e.g.* for digitalis glycosides[4] and alkylphenols[5]. The selectivity in straight-phase adsorption chromatography has been discussed by Snyder[6,7], Soczewinski[8,9] and Scott and Kucera[10], and the retention mechanisms of reversed phases have been considered by several authors, as discussed in ref. 1.

EXPERIMENTAL

Equipment

The liquid chromatograph was from Waters Assoc. (Milford, Mass., U.S.A.), involving a M 6000A pump, a U6K injector system and a M 440 UV detector (254 nm).

Column and packing materials

Prepacked columns (300 mm × 3.9 mm I.D.) were received from Waters Assoc., and the supports investigated were μPorasil, μBondapak NH_2, μBondapak CN (used both in straight- and reversed-phase modes), μBondapak C_{18} and μBondapak alkylphenyl. These supports consist of 10 ± 2 μm irregular particles. Further details concerning the packing materials were kindly supplied by Dr. D. Vivilecchia[11], and these are given in Table I. The μBondapak alkylphenyl material is newly introduced and only one column was available and tested. In other cases at least two columns were investigated. The V_0 values of the columns were in the range 2.5–3.0 ml, which is in good agreement with a total column porosity of *ca.* 0.8.

TABLE I

SUPPORTS USED IN THE PRESENT WORK

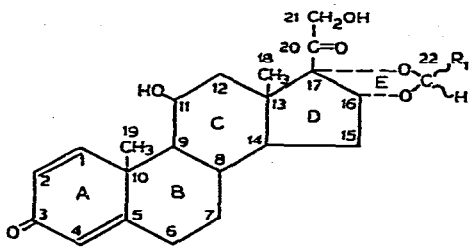
Support	*Surface area (m^2)*	*Surface coverage of functional group (μmol/m^2)*	*Packing weight per column (g)*
μPorasil	320	8	1.7
μBondapak C_{18}	320	1.1	1.9
μBondapak NH_2	320	3.6	1.8
μBondapak CN	320	3.5	1.8

Chromatographic conditions

5–10 μl samples (2 mg/ml), dissolved either in ethanol or in the mobile phase, were injected. The flow-rate in all experiments was 1.0 ml/min, which corresponds to a linear velocity of *ca.* 1.8 mm/sec. Eluents for the straight-phase systems were made from mixtures of hexane (pro analysi) and ethanol (spectroscopic grade), and the mobile phases of the reversed-phase systems were prepared from glass-distilled water and ethanol. All experiments were performed at ambient temperature.

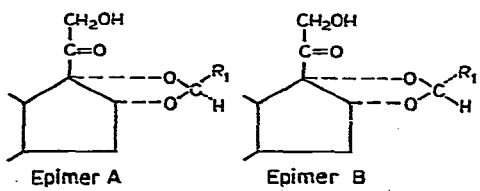
Samples

The corticosteroids investigated are described by the structural formula I ($R_1 = CH_3$, CH_2CH_3, $(CH_2)_2CH_3$ or $(CH_2)_4CH_3$).



I

For each of the acetals a pair of epimers, A and B, is defined by the configuration at C-22. The molecular structures of the epimers of budesonide, $R_1 = (CH_2)_2CH_3$, have been determined by Albertsson *et al.*[12], and have also been discussed briefly in ref. 1.



RESULTS AND DISCUSSION

Plate numbers of straight-phase adsorption and bonded-phase systems

Typical plate numbers for the corticosteroids in the straight- and reversed-phase (ODS) systems were 4300 and 2150, respectively, which corresponds to HETP values of 70 and 140 μm. The plate number of the straight-phase systems is thus a factor two higher than that of the ODS system. For the reversed-phase system it was suggested that the relatively low plate number might originate from combined processes in the mobile phase in which the diffusion of the solute plays an important role[1]. The sample diffusion coefficient is inversely related to the viscosity of the eluent according to the Wilke–Chang equation[13,14]. The differences in efficiency of the re-

versed and straight phases would then partly be a consequence of higher viscosity of ethanol–water mixtures compared with hexane–ethanol mixtures.

Selectivity of straight-phase systems

The selectivities of the straight-phase systems investigated are reflected by the curves in Fig. 1, where log k' is plotted against the number of carbon atoms in the alkyl chain attached to the centre of chirality. The increasing lipophilic character of the molecules, which follows the addition of methylene groups to the alkyl chain, causes a decrease in the capacity factor. The effect, however, levels out as the alkyl chain length increases, and the selectivity with respect to homologues is very low compared with the reversed-phase systems. In straight-phase chromatography interactions between polar groups of the solute and the stationary phase are the main contribution to retention. However, as the polar groups are identical for all the steroids studied, the selectivity may, to a large extent, be governed by weak dispersive solute–solvent interactions in the mobile phase. An influence of solute–solvent interactions on the selectivity is plausible, because the resolution was diminished by adding chloroform, in which the steroids are highly soluble. Alterations of the hexane–ethanol proportions had very little effect on the selectivity.

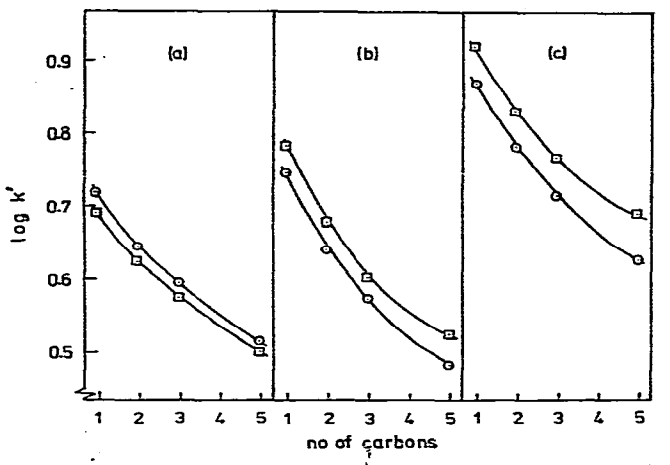


Fig. 1. Log k' plotted against the number of carbons attached to the centre of chirality (C-22) of 16α-hydroxyprednisolone derivatives. Support: (a) μBondapak CN, (b) μPorasil, (c) μBondapak NH_2. Mobile phase: hexane–ethanol (92.5:7.5). Flow-rate: 1.0 ml/min. Solute: (⊙) epimer A and (⊡) epimer B of 16α-hydroxyprednisolone derivatives.

The separation of epimers on different straight-phase supports increased in the order CN, SiOH, NH_2 (Fig. 1). The order corresponds to increasing polarity of the packing materials. This suggests that solute–stationary phase interactions contribute to the separations, as the same mobile phase was used in all experiments. The trend even causes the elution order of the epimers to change in the isopropylcyano system, as for this system epimer B is eluted before epimer A. It is noteworthy that for all the straight-phase systems the separation of epimers is mainly independent of the alkyl chain length at the chiral centre C-22, which is further demonstrated

by Figs. 2 and 3. This is logical, owing to the low selectivity of the systems with respect to the number of methylene groups in the alkyl chain, and it is also compatible with the findings of Scott *et al.*[15]. They prepared diastereomers by reaction between optically pure α-methyl-*p*-nitrobenzylamine and acylic isoprenoid acids with alkyl chains of various lengths. It was suggested that the distance between the chiral centre of the acid and the polar amide group of the derivatives was a determining factor for the degree of separation of the diastereomers in adsorption chromatography systems. For the 16α-hydroxyprednisolone derivatives studied here, the distance between polar groups, *e.g.* carbonyl at C-20, hydroxyl at C-21, oxygens at C-16, C-17 and the centre of chirality at C-22, is approximately independent of the alkyl chain at C-22. Consequently the length of the latter has only a marginal effect on the separation of the epimers. Chromatograms illustrating optimal separations of typical 16α-hydroxyprednisolone acetals in the NH_2 system are shown in Figs. 2 and 3.

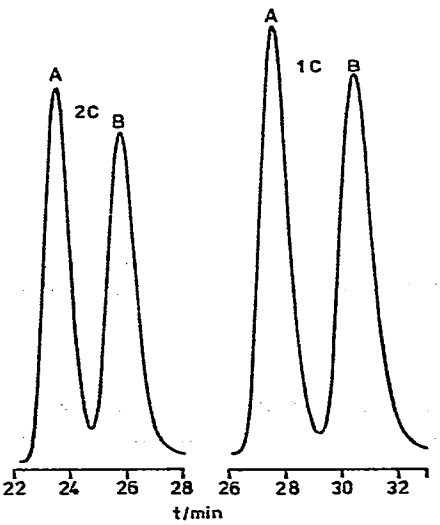


Fig. 2. Separation of 16α-hydroxyprednisolone acetals. Support: μBondapak NH_2. Mobile phase: hexane–ethanol (92.5:7.5). Flow-rate: 1.0 ml/min. Solute: number of carbons of the alkyl chain at C-22 and epimers are given in the figure.

The relative elution order of the epimers suggests that epimer B is more polar than epimer A. This has been proposed from a consideration of the molecular structures of the epimers of budesonide[1]. In epimer A the alkyl chain may be directed either equatorially or axially whereas in epimer B, for steric reasons, it is supposed to be directed only axially with respect to the steroid nucleus. When the chain is oriented axially in the two epimers their structures are very close. These structures are more bulky and probably more polar than epimer A with the chain directed equatorially. Moreover, in the equatorial conformation of epimer A the alkyl chain may sterically interfere with the polar groups at C-16, C-17, C-20 and C-21 and thus slightly shield the polar functional groups. This effect might decrease the polarity of epimer A compared with epimer B and the effect might be more pronounced with

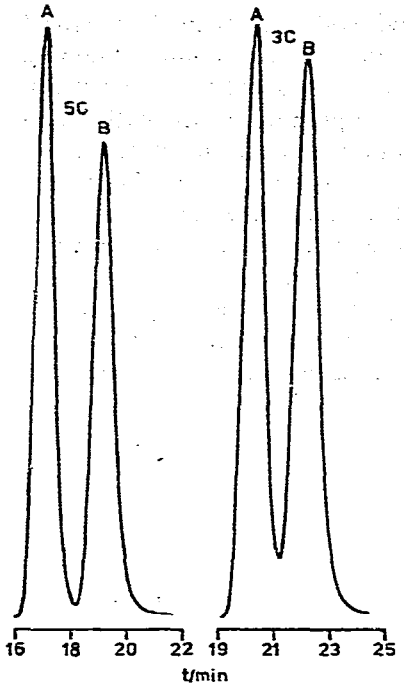


Fig. 3. Separation of 16α-hydroxyprednisolone acetals. Support: μBondapak NH_2. Mobile phase: hexane–ethanol (92.5:7.5). Flow-rate: 1.0 ml/nim. Solute: number of carbons of the alkyl chain at C-22 and epimers are given in the figure.

increasing chain length. This would even, to some extent, enhance the separation of epimers with long chain length, as was found in the silica and amino systems (Fig. 1).

Selectivity of reverse bonded-phase systems

The reversed-phase systems exhibit a more systematic retention behaviour than the straight-phase systems (Fig. 4). Fig. 4 shows that log k' varies linearly with the carbon number of the alkyl chain and that epimer B is eluted before epimer A for all the packing materials. However, the slopes of the lines representing the two epimers are different, *i.e.* the separation of epimers increases strongly with the carbon number in the substituent at C-22. It is also notable that for the two epimers with a methyl group at C-22 there is a slight separation in the alkylphenyl system, whereas negligible and no separation is obtained in the isopropylcyano and ODS systems, respectively. Chromatograms illustrating these features are shown in Figs. 5 and 6. According to Horváth *et al.*[16] the retention in a reversed-phase system is determined by solvophobic interactions between the polar mobile solvent and the non-polar parts of the solute. This gives rise to a high selectivity of the systems with respect to the number of methylene groups in the alkyl chain of the 16α-hydroxyprednisolone acetals compared with the straight-phase adsorption systems, as discussed above. Horváth *et al.* derived an expression for the capacity factor, k', saying that for closely related sub-

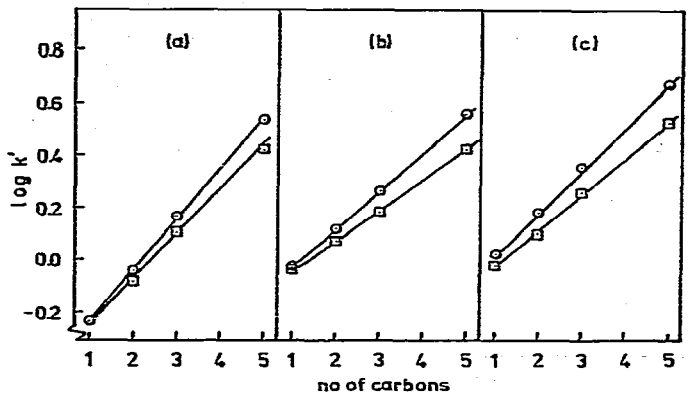


Fig. 4. Log k' plotted against the number of carbons at C-22 of the 16α-hydroxyprednisolone acetals. Support: (a) μBondapak C_{18}, (b) μBondapak CN, (c) μBondapak alkylphenyl. Mobile phase: (a) water–ethanol (44:56); (b) water–ethanol (60:40); (c) water–ethanol (45:55). Solute and flow-rate: see Fig. 1.

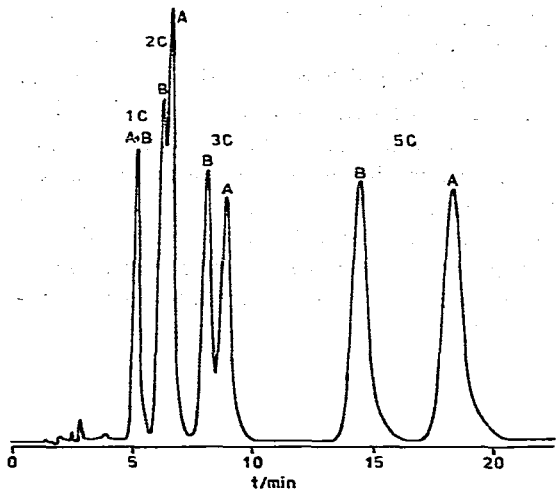


Fig. 5. Chromatogram of 16α-hydroxyprednisolone derivatives. Support: μBondapak C_{18}. Mobile phase: water–ethanol (48:52). Solute and flow-rate: see Fig. 2.

stances, $\log k'$ is linearly related to the contact area, ΔA, between the solute and the C_{18} ligand of an ODS support. Derivation of the approximated Horváth equation with respect to the contact area gives

$$\frac{\mathrm{d} \log k'}{\mathrm{d}\Delta A} = \frac{\gamma}{940.7} \quad (1)$$

where γ is the surface tension of the eluent. Eqn. 1 reflects a change in retention with a change in contact area when a methylene group is added to the alkyl chain. By comparing the slopes obtained from eqn. 1 with experimental slopes of lines like those in Fig. 4, using a hydrocarbon surface area (HCSA) for one methylene group

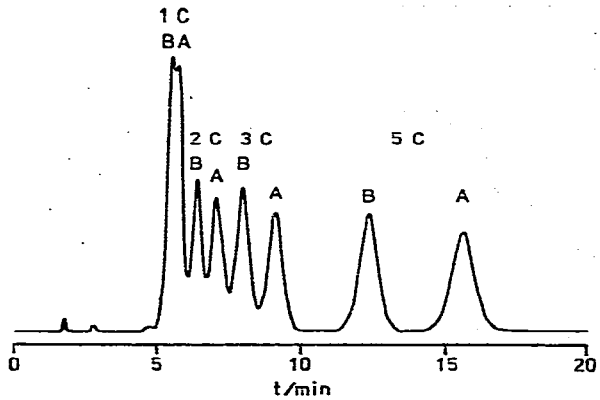


Fig. 6. Chromatogram of 16α-hydroxyprednisolone derivatives. Support: μBondapak alkylphenyl. Mobile phase: water–ethanol (45:55). Solute and flow-rate: see Fig. 2.

of 18.1 Å², the relative contact areas as a percentage of HCSA can be obtained[1]. Values are plotted against the concentration of ethanol in the eluents for the different packing materials (Fig. 7). It is seen that there are variations in the values with the eluent composition, and this is probably due to solvation effects[1]. However, the different levels of the curves indicate, in terms of the Horváth model, that the contact areas in the stationary phase complexes decrease in the order ODS > alkylphenyl > isopropylcyano. This also means that the selectivity increases with increasing lipophilic character of the stationary phase. The larger slopes in the plots of carbon number against log k' (Fig. 4) for epimer A compared with B indicate a closer contact between the alkyl chain of epimer A and the stationary bonded phase. This should occur if there is a flipping over from one conformation in the mobile phase (alkyl chain at C-22 directed axially) to another representing a larger HCSA, in the stationary phase (alkyl chain directed equatorially).

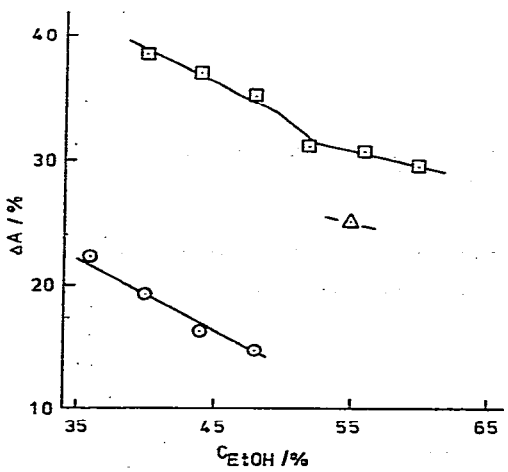


Fig. 7. Relative contact areas in percentage of HCSA for a methylene group of the alkyl chain at C-22 plotted against the concentration of ethanol in aqueous eluents. Support: (⊡) μBondapak C_{18}, (△) μBondapak alkylphenyl, (⊙) μBondapak CN.

Resolution

For closely related substances, *e.g.* for the separation of epimers of corticosteroids, the resolution equation is approximated by[1]

$$R_s = \frac{1}{4}\frac{\alpha - 1}{\alpha}\sqrt{N}\frac{k'}{1 + k'} \quad (2)$$

In Fig. 8 the resolution of epimers is plotted against the carbon number of the alkyl chain at C-22 of the 16α-hydroxyprednisolone acetals. The plots are for $k' = 6$. Eqn. 2 shows that an increase of a factor two in N, which was obtained by changing the system from reversed to straight phase, only marginally influences R_s. Baseline separation for all epimer pairs ($R_s \geqslant 1.5$) is obtained only on the amino system. However, equally good or better separation is obtained in the C_{18}-system for alkyl chains of $\geqslant C_3$, because the separation factor increases strongly with increasing alkyl chain length. In the straight-phase systems the separation factors and resolutions are roughly independent of the alkyl chain length. In these systems the resolution of the epimers increases with the polarity of the adsorption phase, *i.e.* in the order CN, SiOH, NH_2. Comparison of Figs. 2 and 3 with Fig. 5 reveals that for homologues the reversed-phase system is superior to the straight-phase system. The selectivity increases with increasing lipophilic character of the stationary bonded phase, *i.e.* in the order isopropylcyano < alkylphenyl < ODS, which corresponds to an increase in the bristle length, as shown by Karch *et al.*[18] for *n*-alkanes.

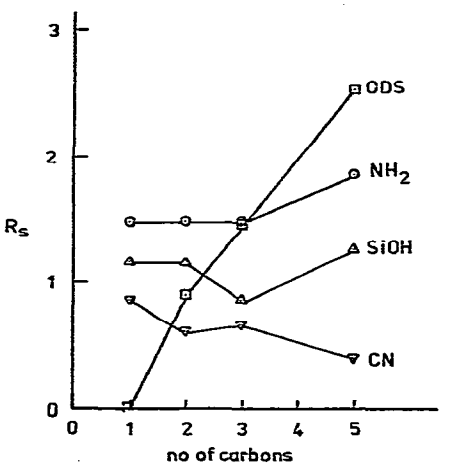


Fig. 8. The resolution of epimers of 16α-hydroxyprednisolone acetals plotted against the number of carbons at the centre of chirality (C-22). Supports are given in the figure, and $k' = 6$ was used in all cases.

The results illustrate that the HCSA of the solute is most important for selectivity of the reversed-phase systems, whereas the polar groups of the packing material and the solute play important roles in the straight-phase systems. Although, among the systems studied, the one based on ODS is best fitted for most separations, at least two systems must be used to obtain optimal resolutions of all solutes studied in the present investigation.

ACKNOWLEDGEMENT

We thank Dr K. Callmer for valuable discussions.

REFERENCES

1 A. Wikby, L. Nilsson and G. Hällsås, *J. Chromatogr.*, 157 (1978) 51.
2 R. L. Brattsand, B. Thuresson af Ekenstam, K. G. Claeson and A. Thalén, *U.S. Pat.*, 3,929,768 (1975).
3 R. L. Brattsand, B. Thuresson af Ekenstam, K. G. Claeson and A. Thalén, *U.S. Pat.*, 3,928,326 (1975).
4 F. Erni and R. W. Frei, *J. Chromatogr.*, 130 (1977) 169.
5 K. Callmer, L.-E. Edholm and B. E. F. Smith, *J. Chromatogr.*, 136 (1977) 45.
6 L. R. Snyder, *Principles of Adsorption Chromatography*, Marcel Dekker, New York, 1968.
7 L. R. Snyder, *Anal. Chem.*, 46 (1974) 1384.
8 E. Soczewinski, *Anal. Chem.*, 41 (1969) 179.
9 E. Soczewinski, *Chromatographia*, 6 (1973) 269.
10 R. P. W. Scott and P. Kucera, *Anal. Chem.*, 45 (1973) 749.
11 D. Vivilecchia, Waters Associates Inc., Milford, Mass., personal communication.
12 J. Albertsson, Å. Oskarsson and C. Svensson, *Acta Crystallogr.*, B34 (1978) in press.
13 L. R. Snyder and J. J. Kirkland, *Introduction to Modern Liquid Chromatography*, Wiley, New York, 1974, p. 491.
14 J. C. Giddings, *Dynamics of Chromatography*, Marcel Dekker, New York, 1965, p. 233.
15 C. G. Scott, M. J. Petrin and T. McCorkle, *J. Chromatogr.*, 125 (1976) 157.
16 C. Horváth, W. Melander and I. Molnár, *J. Chromatogr.*, 125 (1976) 129.
17 S. C. Valvani, S. H. Talkowsky and G. L. Amidon, *J. Phys. Chem.*, 80 (1976) 829.
18 K. Karch, I. Sebestian and I. Halász, *J. Chromatogr.*, 122 (1976) 3.