Journal of Chromatography, 254 (1983) 45–52
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 15,321

REVERSED-PHASE ION-PAIR CHROMATOGRAPHY OF ANTHRACYCLINES

M. A. VAN LANCKER, H. J. C. F. NELIS and A. P. DE LEENHEER*
Laboratoria voor Medische Biochemie en voor Klinische Analyse, Rijksuniversiteit Gent (RUG), Harelbekestraat 72, B-9000 Ghent (Belgium)
(Received August 26th, 1982)

SUMMARY

A new reversed-phase liquid chromatographic separation of anthracyclines on a spherical octadecylsilica material is described. The effect of variation of the content of organic modifier, pH, counter-ion concentration and ionic strength on retention and selectivity has been investigated. There is evidence that, in acidic eluents not containing a counter-ion, mixed retention mechanisms are operative. Solvophobic interactions appear to predominate in water-rich eluents, whereas high organic modifier concentrations favour polar-type retention mechanisms, possibly involving silanol groups. An eluent containing sodium dodecyl sulphate afforded improved efficiency and resolution and was used to resolve eight anthracycline derivatives in one isocratic run.

INTRODUCTION

Anthracyclines are among the most widely used anticancer agents. In particular, adriamycin (doxorubicin) is extensively used for the treatment of a variety of tumours. New semi-synthetic derivatives are currently being developed or already undergoing clinical trial[1,2]. The basic anthracycline structure consists of a tetracyclic quinoid moiety, coupled to an amino-sugar. Reduction of the carbonyl side chain and deglycosylation are the predominant metabolic processes (Fig. 1).

Several high-performance liquid chromatographic (HPLC) methods for separation and quantitation of adriamycin and derivatives have been described. Although a number of straight-phase systems have been proposed[3–7], most workers currently prefer reversed-phase chromatography[8–15].

C_{18} reversed-phase supports[8–11] are gradually replacing shorter-chain-length materials[12,13] or phenyl bonded phases[14,15] for anthracycline chromatography. The mobile phase is generally a methanol–water or an acetonitrile–water mixture, acidified to pH 2–4 (refs. 8–15). In one paper[15] the use of reversed-phase ion-pair chromatography using heptanesulphonate as a counter-ion was described, but found not to be superior over a system without an ion-pairing agent. The substantial polarity difference between glycosylated derivatives and aglycones led some workers to use

0021-9673/83/0000–0000/$03.00

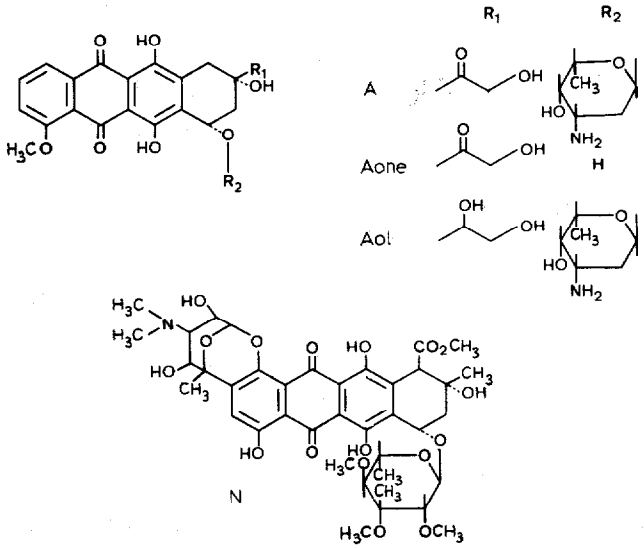


Fig. 1. Structural formulae of adriamycin (A), adriamycinol (Aol), adriamycinone (Aone) and nogalamycin (N).

gradient elution[7,14,15], while other methods were designed to separate only a few products[8,13]. So far, no systematic investigation on the effect of different eluent parameters on retention and resolution of anthracyclines has been carried out.

This paper describes a new reversed-phase system on a spherical octadecylsilica which affords improved efficiency and resolution. The relationship between the capacity ratios, k', of four adriamycin analogues and organic modifier concentration, pH, sodium dodecyl sulphate (SDS) content and ionic strength have been studied. The ion-pairing approach has some distinct advantages and allows separation of parent compounds and both more and less polar metabolites in one run, without the need for gradient elution.

EXPERIMENTAL

Chemicals

All chemicals were of analytical grade and were purchased from E. Merck (Darmstadt, G.F.R.) except SDS which was obtained from Fluka (Buchs, Switzerland). Adriamycin and metabolites were a gift from F. Arcamone (Farmitalia, Milan, Italy), nogalamycin from S. J. Stein (Upjohn, Kalamazoo, MI, U.S.A.) and daunomycin and metabolites from G. Jolles (Rhône-Poulenc, Paris, France).

Apparatus

The chromatographic system consisted of two N60 syringe-type pumps (Varian 8500; Varian, Walnut Creek, CA, U.S.A.), a Valco sample valve injector (Valco Instrument Co, Houston, TX, U.S.A.) equipped with a 10-μl loop and a Varichrom variable wavelength detector (Varian) set at 480 nm. Columns were of stainless steel (15 × 0.32 cm I.D. or 25 × 0.46 cm I.D.) with Valco connectors.

The packing material was Spherisorb 5S-ODS (Phase Separations, Queensferry, Great Britain). A 25 × 0.46 cm prepacked column was obtained from Chrompack (Merksem, Belgium). It was used for the final separation of eight anthracyclines, under optimized conditions. Other columns (15 × 0.32 cm) were home-packed using a slurry technique under the following conditions: slurry medium, carbon tetrachloride–methanol (9:1, v/v); slurry concentration, 10% (w/v); packing pressure, 41 MPa (6000 p.s.i.); pressurizing liquid, acetonitrile.

Test compounds

Test substances included adriamycin (doxorubicin-A), its slightly more polar metabolite adriamycinol (A-ol), the aglycone adriamycinone (A-one) and nogalamycin (N). The latter lacks the amino group on the sugar, but has a tertiary amino group on the tetracyclic structure (Fig. 1).

Procedure

The influence of mobile phase strength on the capacity ratios of test compounds was investigated by stepwise change of the water–acetonitrile ratio, keeping pH and ionic strength constant by addition of 0.02 *M* phosphate buffer (pH 2.5). Logarithms of capacity ratios, k', were plotted against the phase ratio, q, of water–acetonitrile. The mobile phase linear velocity was 0.15 cm sec^{-1} throughout.

Eluents were adjusted to different pH values by addition of phosphoric acid or 0.02 *M* phosphate buffer. The ionic strength was kept constant at 0.03, using sodium sulphate.

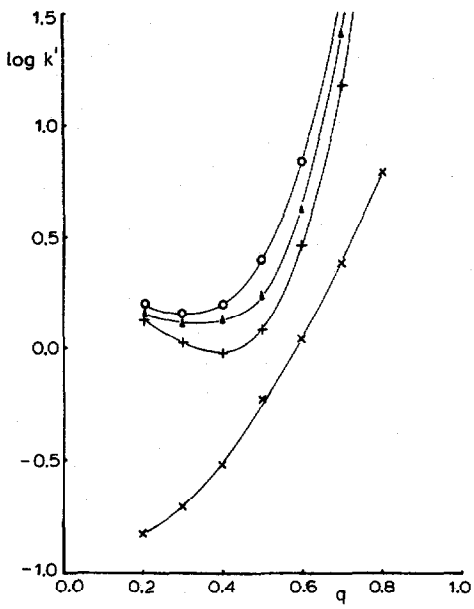
In order to study the effect of counter-ion concentration, increasing amounts of SDS were added to the eluent. The sodium concentration was kept constant at 0.06 *M* by addition of sodium sulphate.

RESULTS AND DISCUSSION

Chromatography of anthracyclines in the absence of specific counter-ions

Reversed-phase systems for anthracyclines have previously been based primarily on acidic mobile phases[8–15]. Eluents consisting of water–acetonitrile–phosphoric acid are often used[10,12,13], but may be accompanied by low chromatographic efficiency and poor peak shape[12]. In our experience, with this type of eluent, spherical packing materials, unlike irregular ones, yield higher performance in terms of peak symmetry and theoretical plate counts. We now show that adriamycin and analogues can be conveniently chromatographed on a 5-μm spherical octadecyl silica material (Spherisorb ODS).

The peculiar retention behaviour displayed by these derivatives suggests that a mixed interaction mechanism is operative. Fig. 2 shows the effect of increasing solvent strength on the capacity ratios of several test compounds. The plot is not linear (except for the aglycone), as predicted on the basis of solvophobic theory[16,17], but goes through a distinct minimum. This observation is essentially consistent with similar findings reported earlier for adriamycin and adriamycinol[12] as well as for tetracyclines[18] and crown ethers[19,20] on other reversed-phase materials. It may be rationalized by assuming that, in addition to hydrophobic interactions, interactions with silanol groups are operative. The latter are likely to predominate in water-lean



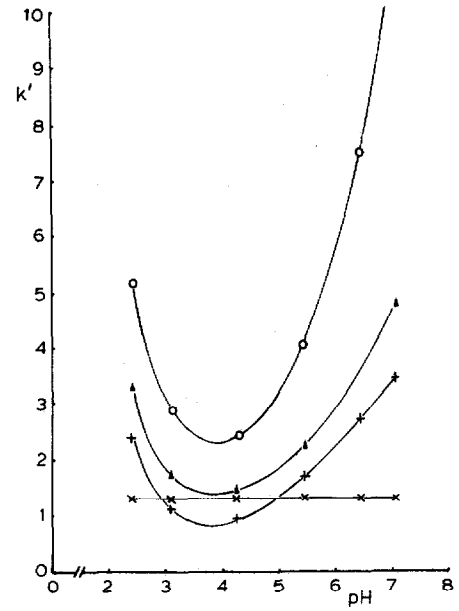


Fig. 2. Effect of mobile phase strength on retention. Column: 15 × 0.32 cm. Packing: Spherisorb, 5-μm ODS. Eluent: 0.02 *M* H_3PO_4 in acetonitrile–water (pH 2.5). Linear velocity 0.1 cm sec^{-1}. Compounds: ▲, adriamycin; +, adriamycinol; ×, adriamycinone; ○, nogalamycin.

Fig. 3. Effect of pH on retention. Column packing, flow-rate and compounds as in Fig. 2. Eluent: acetonitrile–water (40:60), buffered as described in text.

eluents (low values of *q*), whereas hydrophobic interactions predominate in water-rich eluents. The theory of such a dual retention mechanism has been presented by several workers[19,24]. However, this behaviour has also been attributed to adduct formation with solvent molecules[12].

The *k' versus* pH plot has a similar non-linear shape and displays a minimum at around pH 4 (Fig. 3). Again, such a relationship is unexpected. In acidic media, the amino group is protonated and one would expect the retention on a non-polar stationary phase to be minimal, provided no polar interactions are involved[21]. Therefore, at low pH, the plot is difficult to rationalize only on the basis of solvophobic theory, and again the occurrence of polar interactions should be considered.

A strong argument supporting the hypothesis of the interactions with free silanol groups is that only the more polar compounds exhibit this anomalous retention behaviour. The amino and/or alcoholic groups tend to interact readily with silanols[20]. The plot of log *k' versus q* for adriamycinone (which lacks such groups) is indeed close to linearity (Fig. 2). Furthermore, because of its inability to undergo polar interactions the aglycone elutes before the other analogues, despite its higher lipophilicity. This phenomenon is most pronounced in water-lean eluents, which favour polar interactions with the glycosylated derivatives. It is therefore not surprising that maximum selectivity for the pair adriamycin/adriamycinone is obtained at low water contents (Fig. 2). The selectivity is gradually lost at higher water concentrations, when silanols are masked and hydrophobic interactions become predominant for both compounds. Additional support for this hypothesis can be derived from Fig.

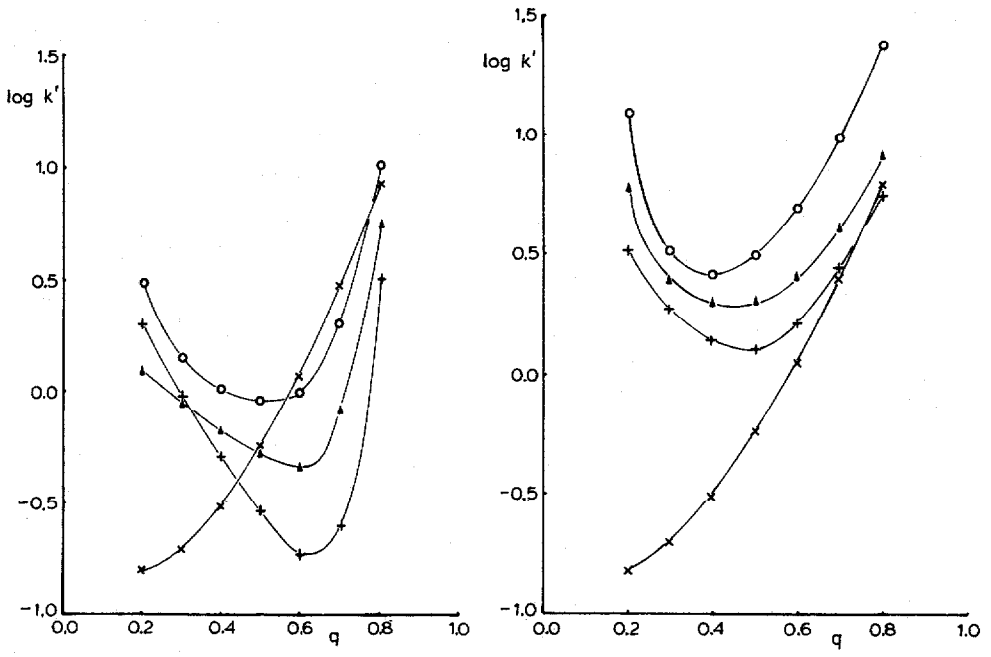


Fig. 4. As Fig. 2, but eluent containing also 1.5 m*M* nonylamine.

Fig. 5. As Fig. 2, but eluent containing also 1.5 m*M* SDS.

4, which shows plots of log k' *versus* q obtained with an eluent containing nonylamine. Amines are known to deactivate accessible silanol groups and, hence, will counteract interactions with silanol groups more or less efficiently[20]. Although plots are not linear, a significant reduction in retention was observed for all compounds except adriamycinone. This resulted in a simultaneous decrease in selectivity for the pair adriamycin/adriamycinone, to a point where even a reversal of the elution order occurred. At relatively high water concentrations ($q = 0.6$), all other derivatives eluted before adriamycinone, in decreasing order of polarity. This indicates that hydrophobic interactions are predominant.

As to the nature of the chemical function(s) that may be involved in silanol interactions, the amino group on the sugar moiety appears to be the most likely candidate. However, if only the amino groups were involved, the plots of log k' *versus* q should again approach linearity upon addition of a suitable ion-pairing agent[20]. In an acidic medium, a suitable counter-ion should block the amino function and thus prevent its interaction with silanols. As can be judged from Fig. 5, only a partial effect was noted upon SDS addition. Retention in water-lean eluents is significantly decreased, but it is enhanced elsewhere. Higher concentrations of SDS had no appreciably greater effect on the shape of the plots, albeit overall retention was enhanced. We believe this is due to the presence, besides the amino function, of other polar groups, *e.g.*, hydroxyl groups, that might underly other polar interactions.

Ion-pair chromatography

Although our results show that anthracyclines can be chromatographed in

acidic eluents containing no additives, the overall efficiency of the system remained below current standards. Peak symmetry could only be obtained in a narrow pH range, *i.e.*, 2–3, as peak shape rapidly deteriorated at higher pH values. Our observations are essentially consistent with the results reported previously[12].

Addition of sodium dodecyl sulphate had a beneficial effect on the system's general performance. The retention of all derivatives except adriamycinone substantially increased, as shown in Table I. Also, at the same time, the resolution, selectivity and overall efficiency greatly improved and the useful pH range was extended to 5. Of the different alkyl sulphates and sulphonates tried, SDS proved to be superior. Sulphates with shorter alkyl chains yielded lower efficiencies, whereas sodium heptanesulphonate and dodecyl sulphonate resulted in tailing, even at low pH.

TABLE I

CAPACITY RATIO, k', THEORETICAL PLATE NUMBER, N, AND RESOLUTION, R_s, FOR ADRIAMYCIN AND VARIOUS DERIVATIVES

$k' = (t_R - t_0)/t_0$, where t_R = retention time of the compound and t_0 = retention time of the solvent; $N = 5.53 \cdot (t_R/w_{1/2})^2$, where $w_{1/2}$ = half-height width of the peak; $R_s = 2(t_{R2} - t_{R1})/(w_1 + w_2)$, where t_{R1}, t_{R2} = retention times of compounds 1, 2, and $w_{1/2}$ = base-width of the peaks.

	Adriamycinol			*Adriamycinone*			*Adriamycin*			*Nogalamycin*		
	k'	N	R_s	k'	N	R_s	k'	N	R_s	k'	N	R_s
Without SDS	0.8	860	1.9	1.7	790	0.5	1.4	760	–	3.0	960	3.2
With SDS	7.4	1020	12.8	1.8	800	3.8	12.7	1280	–	20.4	1330	4.1

There is ample evidence to indicate that, in the presence of SDS, ion-pair formation or dynamic ion exchange with glycosylated anthracyclines takes place. The capacity ratios k' of the glycosylated derivatives gradually increased when the SDS concentration was raised, reaching a maximum at 30–40 mM, whereas retention of the aglycone remained unaffected throughout the whole range of SDS concentrations studied (Fig. 6). Similar behaviour has been observed for a variety of ion-pairing agents and compounds[21,22,25]. The decrease in retention that occurs beyond a critical SDS concentration may be attributable to micelle formation in the detergent. However, this phenomenon may also occur at values that were well below the critical micelle concentration[22].

Increasing the ionic strength while keeping other parameters constant lowers overall retention (Fig. 7), a phenomenon which is often observed in ion-pair chromatography[23]. Again, variation of ionic strength did not influence the retention of adriamycinone. Since ion-pair formation with SDS requires a positively charged amino group, it is obvious that the aglycone is unable to undergo such an interaction.

Separation of a mixture of anthracyclines

A separation of eight anthracyclines under optimized conditions on a Spherisorb ODS column (25 × 0.46 cm) is shown in Fig. 8. Theoretical plate numbers as high as 10,000 were obtained for adriamycin, comparing favourably with efficiencies reported for other systems. No gradient is required to separate the more and less polar derivatives. Since deglycosylation is a common metabolic route, it is advan-

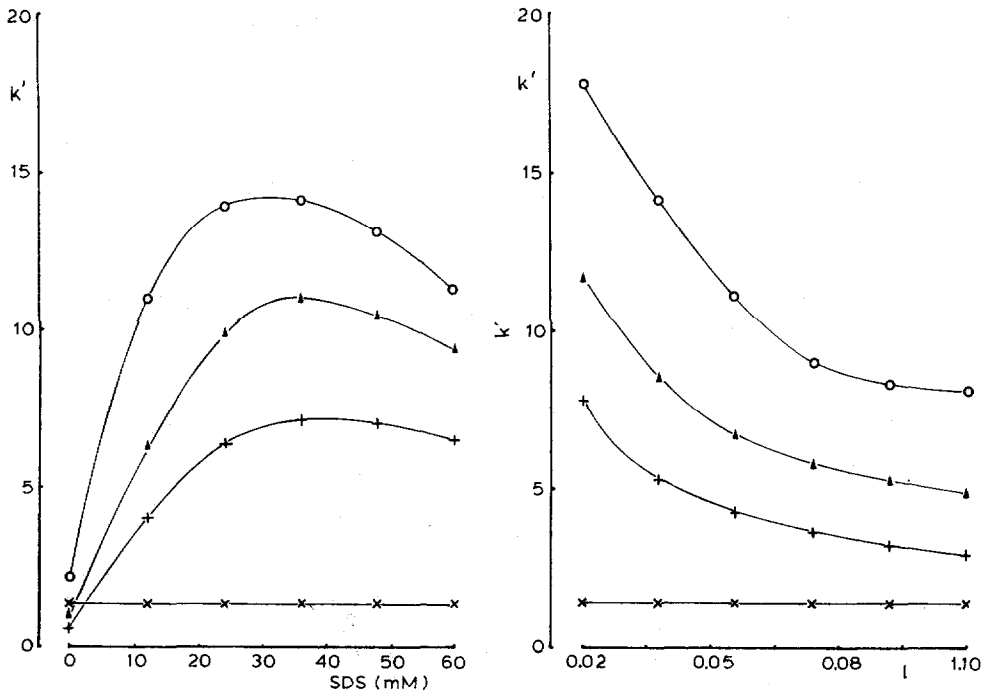


Fig. 6. Changes in capacity ratios as a function of SDS concentration. Column, packing, linear velocity and compounds as in Fig. 2. Eluent: 0.02 *M* H_3PO_4 in acetonitrile–water (40:60). Ionic strength kept constant at 0.03 by means of sodium sulphate.

Fig. 7. Influence of ionic strength on retention in the presence of an ion-pairing agent. Column, packing, linear velocity and compounds as in Fig. 2. Eluent: 0.01 *M* SDS and 0.02 *M* H_3PO_4 in acetonitrile–water (40:60).

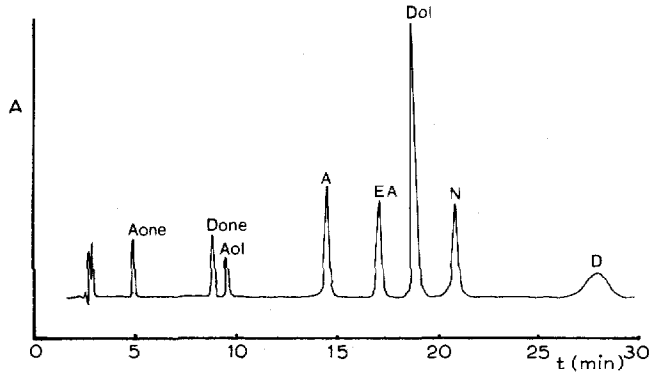


Fig. 8. Chromatogram of a mixture of anthracyclines. Column: 25 × 0.46 cm. Packing: Spherisorb, 5-μm ODS. Eluent: 0.01 *M* SDS and 0.02 *M* H_3PO_4 in acetonitrile–water (40:60) (pH 2.5). Linear velocity: 0.1 cm sec^{-1}. Compounds: Aone = adriamycinol; N = nogalamycin; D = daunomycin. Amounts injected; *ca.* 10 ng.

tageous that the aglycone species are eluted before the parent compounds in this system. Yet, whenever necessary, *e.g.*, for identification purposes, this elution order can be reversed by replacing the ion-pairing agent with an amine (although in this case the chromatographic conditions are not optimal). Since adriamycin and major metabolites were easily resolved, this new system has great potential as a basis for an assay of those compounds in a variety of biological materials.

ACKNOWLEDGEMENTS

This work was in part supported by the National Fund for Scientific Research (NFWO) through a grant to the second author.

REFERENCES

1 S. T. Crooke and S. D. Reich (Editors), *Anthracyclines*, Academic Press, New York, 1980.
2 F. Arcamone (Editor), *Doxorubicin*, Academic Press, New York, 1981.
3 R. Baurain, D. Deprez-De Campeneere and A. Trouet, *Anal. Biochem.*, 94 (1979) 112.
4 J. H. Peters and J. F. Murray, Jr., *J. Liquid Chromatogr.*, 2 (1979) 45.
5 H. G. Barth and A. Z. Conner, *J. Chromatogr.*, 131 (1977) 375.
6 S. Shinozawa and T. Oda, *J. Chromatogr.*, 212 (1981) 323.
7 M. Israel, W. J. Pegg, P. M. Wilkinson and M. B. Garnick, *J. Liq. Chromatogr.*, 1 (1978) 795.
8 A. C. Haneke, J. Crawford and A. Aszalos, *J. Pharm. Sci.*, 70 (1981) 1112.
9 A. J. Quattrone and D. F. Ranney, *J. Anal. Toxicol.*, 4 (1980) 12.
10 R. N. Pierce and P. I. Jatlow, *J. Chromatogr.*, 164 (1979) 471.
11 J. F. Strauss, R. L. Kitchens, V. W. Patrizi and E. P. Enkel, *J. Chromatogr.*, 221 (1980) 139.
12 S. Eksborg, *J. Chromatogr.*, 149 (1978) 225.
13 S. Eksborg, H. Ehrsson and I. Andersson, *J. Chromatogr.*, 164 (1979) 479.
14 J. J. Langone and H. Van Vunakis, *Biochem. Med.*, 12 (1975) 283.
15 P. A. Andrews, D. E. Brenner, F. E. Chou, H. Kubo and N. R. Bachur, *Drug Metab. Dispos.*, 8 (1980) 152.
16 B. L. Karger, J. R. Grant, A. Hartkopf and P. H. Weiner, *J. Chromatogr.*, 128 (1976) 65.
17 M. J. M. Wells and C. R. Clark, *J. Chromatogr.*, 235 (1982) 31.
18 S. Eksborg and B. Ekqvist, *J. Chromatogr.*, 209 (1981) 161.
19 A. Nahum and Cs. Horváth, *J. Chromatogr.*, 203 (1981) 53.
20 K. E. Bij, Cs. Horváth, W. R. Melander and A. Nahum, *J. Chromatogr.*, 203 (1981) 65.
21 Cs. Hováth, W. Melander and I. Molnar, *Anal. Chem.*, 49 (1977) 142.
22 R. S. Deelder and J. H. M. van den Berg, *J. Chromatogr.*, 218 (1981) 327.
23 Cs. Horváth, W. Melander, I. Molnar and P. Molnar, *Anal. Chem.*, 49 (1977) 2295.
24 L. R. Snyder, J. W. Dolan and J. R. Grant, *J. Chromatogr.*, 165 (1979) 3–30.
25 J. M. Knox and G. R. Laird, *J. Chromatogr.*, 122 (1976) 17–34.