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DYNAMIC CATION-EXCHANGE SYSTEMS FOR RAPID SEPARATIONS OF NUCLEOBASES AND NUCLEOSIDES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The retention behaviour of nucleobases and nucleosides in dynamic cation-exchange systems, consisting of a hydrophobic support as the stationary phase and water–ethanol mixtures containing small amounts of sodium dodecylsulphate as anionic detergent as the mobile phase was investigated.

The retention of nucleobases and nucleosides can be influenced over a wide range by variation of the pH and the concentration of the ethanol, anionic detergent and counter ion in the eluent. With respect to separation speed and selectivity, these dynamic cation-exchange systems are in many instances superior to conventional ion-exchange and reversed-phase systems. It is shown that, by optimizing the different retention parameters, the separation of fourteen nucleobases and nucleosides, simultaneously and under isocratic conditions, can be achieved in *ca.* 6 min. The performance of the phase system is demonstrated by the analysis of a calf thymus DNA hydrolysate.

INTRODUCTION

Among the various chromatographic techniques[1–3], column liquid chromatography has been a preferred method for analysing nucleobases and nucleosides. A particular advantage of liquid chromatography is the possibility of analysing directly the hydrolysed biological material, in contrast with gas chromatography, where volatile derivatives are required[4,5].

Until now, ion-exchange[3,6–8] and reversed-phase liquid chromatography[9,10] have been the most frequently applied separation methods for these nucleic acids.

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Although satisfactory for many applications, these phase systems sometimes have limitations with respect to separation speed and selectivity.

In recent years, different modes of ion-pair chromatography[11–16] have proved to be a useful alternative or supplement for the separation of ionizable substances such as sulphonic acids[12,14,15], catecholamines[17,18] and amino acids[19].

Ion-pair chromatography has also been tested for the separation of nucleic acid constituents and their analogues. Thus, for nucleotides[20,21], nucleobases[21], nucleosides[22], 5-fluorouracil nucleotides[22] and azapurines[21], ion-pair chromatography with quaternary ammonium salts has been applied. For the separation of nucleobases and nucleosides, camphorsulphonic acid[20] and heptanesulphonic acid[23] have been applied as pairing ions. In all of these studies only a few naturally occurring nucleobases or nucleosides were used. However, for the characterization of the different DNA and RNA types[24] or for the recognition of alterations in the purine and pyrimidine metabolic pathways, caused by some genetic diseases[25–27], there is a need for a rapid method of determining these nucleobases and their nucleosides. For the separation of these compounds in one run, dynamic anion exchange is not the method of choice in practice because of the high pH of the mobile phase needed to obtain ion pairing for all solutes. For this separation problem, dynamic cation-exchange chromatography is more promising.

In this paper we discuss a very efficient dynamic cation-exchange system with sodium dodecylsulphate as ion-pairing agent for extremely fast isocratic separations of eight naturally occurring nucleobases and many of their nucleosides in one run.

EXPERIMENTAL

Apparatus

The liquid chromatograph consisted of a reciprocating pump (Orlita type AE-10-4.4), a Bourdon-type manometer, a high-pressure injection valve (Rheodyne 7105) equipped with a sample loop of 20 μl and a Zeiss PMQ II UV spectrophotometer (260 nm). The stainless-steel columns had an I.D. of 4.5 mm and a length of 150 mm.

Materials

All solvents and chemicals were of analytical-reagent grade and used without any further pre-treatment. The alkyl-modified silica used as the column support was Hypersil ODS (Shandon, Great Britain), mean particle size 5 μm. The nucleobases and nucleosides were obtained from Sigma (St. Louis, MO, U.S.A.) and Merck (Darmstadt, G.F.R.). The following abbreviations for these substances are used: uracil (Ura); cytosine (Cyt); Thymine (Thy); adenine (Ade); guanine (Gua); hypoxanthine (Hyp); Xanthine (Xan); Uridine (Urd); cytidine (Cyd); adenosine (Ado); guanosine (Guo); Inosine (Ino); xanthosine (Xao); thymidine (dThy); and 5-methylcytosine (5-Cyt).

Procedures

The HPLC columns were packed according to the procedure recommended by the manufacturer of the C_{18} support (Shandon). The columns were washed with 100 ml of methanol and then equilibrated with the eluent until constant retention of the compounds under investigation was obtained.

RESULTS AND DISCUSSION

In dynamic ion-exchange systems one uses alkyl-modified silicas as the stationary phase and aqueous–organic solvent mixtures containing an ionic detergent as the mobile phase. Owing to the hydrophobic part of its molecule, such a detergent is strongly adsorbed on to the hydrophobic column packing and has the ability to exchange its associated counter ion[15,19] (*i.e.*, it can act as an ion exchanger). Such dynamic (solvent-generated) ion-exchange systems have definite advantages over conventional resin ion exchangers, such as excellent exchange kinetics and pressure-stable packings, which allows high separation speeds with highly efficient columns. Moreover, the type and capacity of the ion exchanger can be varied without changing the column packing (*i.e.*, via the mobile phase).

For the investigation of the applicability of dynamic ion-exchange chromatography for the separation of nucleobases and nucleosides, sodium dodecylsulphate (SDS) was chosen as a detergent to generate a cation exchanger on the hydrophobic support. In order to explore fully the possibilities of this dynamic cation-exchange system for nucleobases and nucleosides, the influence of the pH and the concentration of the organic modifier, counter ion and SDS of the mobile phase on the capacity ratio (k'_i) was systematically investigated.

Fig. 1 shows the effect of the addition of SDS to the mobile phase on the retention of a number of nucleobases and nucleosides at constant pH and ethanol and Na^+ concentrations. It can clearly be seen that after addition of only 1.0 g/l of SDS, remarkably different retention behaviour is found for a number of compounds owing to additional retention via cation exchange. The order of elution of nucleobases and nucleosides on this dynamic cation-exchange system is similar to that found on cation-exchange resins[3,6], but is significantly different to the order obtained in reversed-phase elution chromatography[9,10]. The capacity ratio of most solutes increases with increasing amount of SDS and passes a maximum at about 0.1 % (w/w) of SDS. This dependence of k'_i on the SDS concentration in the mobile phase coin-

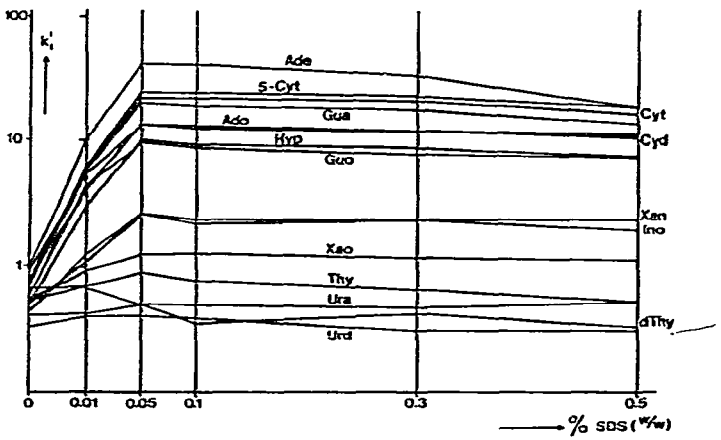

Fig. 1. Effect of the addition of SDS to the mobile phase on the capacity ratio (k'_i) of nucleobases and nucleosides. Stationary phase: Hypersil ODS. Mobile phase: 0.05 *M* $HC.O_4$–ethanol (9:1, v/v) + SDS (0–0.5%).

cides with the shape of the adsorption isotherm of SDS on the stationary phase[14,19]. Roughly, the average retention of the compounds discussed approaches constancy when the SDS concentration is >0.1 %. However, minor variations occur, which can be partly explained by solute–SDS micelles interaction in the mobile phase[15]. Fig. 1 shows that in some instances fine tuning of the selectivity can be accomplished by the choice of the SDS concentration.

The influence of the pH of the mobile phase on k_i' was investigated at constant SDS, ethanol and counter ion [Na^+] concentration, and is shown in Fig. 2. On going from low to high pH, the k_i' values of Ade, Ado, Gua, Guo, Cyd and Hyp show maxima in the pH region 2–3. For Xan and Xao no maximum appears in this pH region but the k_i' values still decrease with increasing pH. The k_i' values of the aforementioned solutes decrease sharply at higher pH and tend to become constant at pH > 6. For Thy, Ura and Urd pH seems to have hardly any influence on k_i' in the investigated pH range of 1–7. The pH of the mobile phase is a valuable parameter for influencing the absolute and relative retentions of nucleobases and nucleosides in dynamic cation-exchange systems.

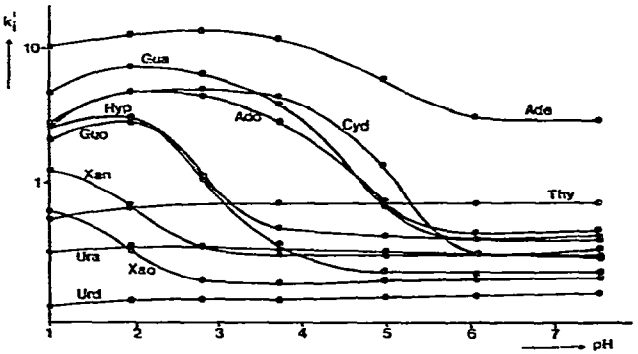

Fig. 2. Effect of the pH of the mobile phase on k_i' of nucleobases and nucleosides. Stationary phase: Hypersil ODS. Mobile phase: water–ethanol (9:1, v/v) + 0.05 *M* Na_3PO_4 + 0.72% (w/w) SDS, pH adjusted with 5 *M* $HClO_4$.

The dependence of the capacity ratio of nucleobases and nucleosides on the volume percentage of ethanol is shown in Fig. 3. Over the ethanol content region investigated (2–30%, v/v) log k_i' for all solutes increases linearly with decreasing volume percentage of ethanol, but with different slopes. This suggests that, apart from retention via cation exchange, physical distribution also contributes significantly to the overall retention of some solutes[15]. The strong influence of the concentration of the ethanol on the degree of retention can be used in gradient elution[19].

The retention of solute ions in dynamic ion-exchange systems can be influenced reasonably predictably by the concentration of the counter ion in the mobile phase, as in conventional ion exchange[15,19]. This is demonstrated for a number of nucleobases and nucleosides in Fig. 4, using Na^+ as the counter ion. It can be seen that $1/k_i'$ is directly proportional to the amount of Na^+ added to the mobile phase over the range 10–150 m*M* of Na^+. As the retention in such dynamic ion-exchange systems is often the result of a mixed mechanism (physical distribution and ion exchange), significant

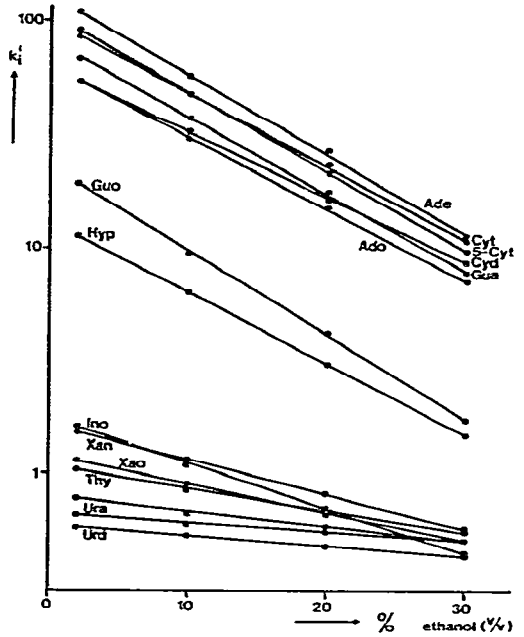

Fig. 3. Effect of the concentration of ethanol on k_i' of nucleobases and nucleosides. Stationary phase: Hypersil ODS. Mobile phase: 0.006 *M* $HClO_4$–ethanol (2–30%, v/v) + 0.72% (w/w) SDS, pH = 2.6.

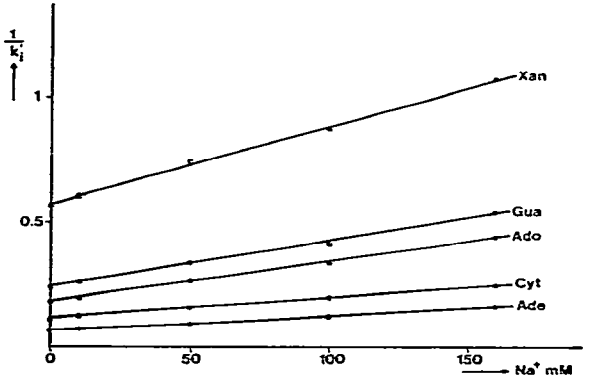

Fig. 4. Dependence of k_i' of nucleobases and nucleosides on the counter ion (Na^+) concentration in the mobile phase. Stationary phase: Hypersil ODS. Mobile phase: 0.1 *M* $HClO_4$–ethanol (9:1, v/v), pH = 1.0 + 0.1% (w/w) SDS + $NaClO_4$ (0–160 m*M*).

selectivity changes can be effected by variation of the counter ion concentration, depending on the relative magnitude of these separate distribution processes[12,15,19]. The counter ion concentration can be used as a parameter to produce gradients.

Apart from the retention characteristics of the nucleobases and nucleosides, the column efficiency was also measured as a function of the mobile phase composition. It was found that for all solutes the theoretical plate height (*H*) varies very little with the composition of the mobile phase. This is in contrast with results ob-

tained with conventional resin ion exchangers[6,8]. The plate height for solutes with $k_i' > 3$ ranges between 25 and 30 μm. For solutes with $k_i' < 3$ the plate height was larger, probably because of a significant contribution of the external peak broadening to the overall peak width.

For a few mobile phase compositions the dependence of H on the linear velocity, $\langle v \rangle$, was also measured. These measurements show that the H value ranges between 20 μm at $\langle v \rangle = 0.5$ mm/sec and 30 μm at $\langle v \rangle = 8$ mm/sec, indicating the excellent mass transfer in these dynamic cation-exchange systems. Further, the chromatographic characteristics of the columns did not change significantly over a 6-months period.

The results of the investigation of dynamic cation-exchange chromatography with SDS (Figs. 1–4) show that many parameters are available for adapting the mobile phase composition to a particular separation problem. The optimal mobile phase composition for a rapid separation of the nucleobases and nucleosides simultaneously and under isocratic conditions (still a difficult problem) can easily be determined from these figures. A low pH, about 10 % of ethanol and 0.1 % of SDS seems to be a good choice, as is demonstrated in Fig. 5, which shows the separation of a test mixture of eight nucleobases and seven nucleosides. Only Ura and dThy are not resolved with this phase system. However, the separation of this pair of solutes might be improved by small variations in pH or ethanol, SDS or Na^+ concentration. Further, the type of organic modifier and anionic detergent can have a significant effect on the retention characteristics of cationic compounds[19], but this was not investigated in this study.

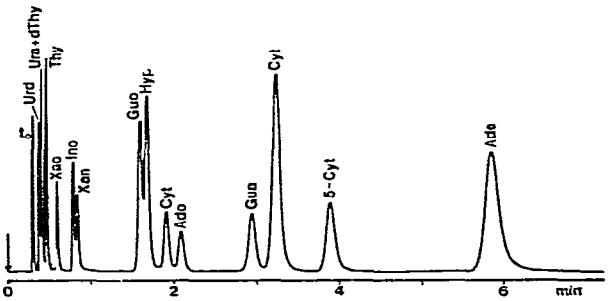

Fig. 5. Rapid separation of a test mixture of nucleobases and nucleosides under isocratic conditions. Stationary phase: Hypersil ODS. Mobile phase: 0.1 M $HClO_4$–ethanol (9:1, v/v) + 0.1 % (w/w) SDS. Ambient temperature; $\Delta P = 170$ bar; $\langle v \rangle = 8$ mm/sec.

The applicability of the described dynamic cation-exchange system for biological samples is demonstrated in Fig. 6, which shows the analysis of a number of nucleobases in a calf thymus DNA hydrolysate.

CONCLUSIONS

Dynamic cation-exchange chromatography, with sodium dodecylsulphate as anionic detergent, is suitable for the separation of nucleobases and nucleosides. Owing to the excellent kinetics of the phase system and the large number of param-

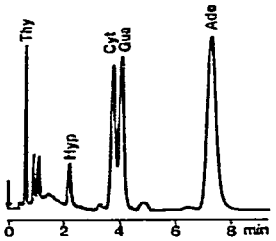

Fig. 6. Analysis of nucleobases in calf thymus DNA hydrolysate. Conditions as in Fig. 5 except ΔP = 130 bar and $\langle v \rangle$ = 6 mm/sec. Hydrolysis: 0.2 g of calf thymus DNA Type I (Sigma) was heated for 16 h at 80°C with 15 ml of 2 *M* HCl, and 1 ml of the supernatant was diluted with 9 ml of water–ethanol (8:1) and 20 μl of the resulting solution were injected on to the column.

eters available for influencing the retention, such as the pH and organic modifier, SDS or counter ion concentration in the mobile phase, it was possible to separate simultaneously almost all nucleobases and their nucleosides under isocratic conditions in 6 min. This result contrasts favourably, with respect to speed and selectivity, to the results obtained with conventional ion-exchange and reversed-phase liquid chromatography. Future work in our laboratory will be devoted to the applicability of these dynamic cation-exchange systems for the separation of modified nucleosides.

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REFERENCES

1 E. Lederer and M. Lederer, *Chromatography, A Review of Principles and Applications*, Elsevier, Amsterdam, 2nd ed., 1957, Ch. 33.
2 W. E. Cohn, in E. Heftman (Editor), *Chromatography*, Reinhold, New York, 2nd ed., 1967, Ch. 22.
3 R. P. Singhal, *Separ. Purif. Methods*, 3 (1974) 339.
4 C. W. Gehrke and C. D. Ruyle, *J. Chromatogr.*, 38 (1968) 473.
5 C. W. Gehrke and A. B. Patel, *J. Chromatogr.*, 123 (1976) 335.
6 Cs. Horváth, *Methods Biochem. Anal.*, 21 (1973) 79–154.
7 P. R. Brown, S. Bobick and F. L. Hanley, *J. Chromatogr.*, 99 (1974) 587.
8 R. Eksteen, J. C. Kraak and P. Linssen, *J. Chromatogr.*, 148 (1978) 413.
9 P. R. Brown, A. M. Krstulovic and R. A. Hartwick, *Advan. Chromatogr.*, 18 (1980) 101.
10 C. W. Gehrke, K. C. Kuo, G. E. Davis, R. D. Suits, T. P. Waalkes and E. Borek, *J. Chromatogr.*, 150 (1978) 455.
11 S. Eksborg and G. Schill, *Anal. Chem.*, 45 (1973) 2092.
12 J. C. Kraak and J. F. K. Huber, *J. Chromatogr.*, 102 (1974) 333.
13 B. A. Persson and B. L. Karger, *J. Chromatogr. Sci.*, 12 (1974) 521.
14 J. H. Knox and G. R. Laird, *J. Chromatogr.*, 122 (1976) 17.
15 C. P. Terweij-Groen, S. Heemstra and J. C. Kraak, *J. Chromatogr.*, 161 (1978) 69.
16 *PIC*, Waters Assoc., Milford, MA, 1975.
17 J. H. Knox and J. Jurand, *J. Chromatogr.*, 125 (1976) 89.
18 J. P. Crombeen, J. C. Kraak and H. Poppe, *J. Chromatogr.*, 167 (1978) 219.
19 J. C. Kraak, K. M. Jonker and J. F. K. Huber, *J. Chromatogr.*, 142 (1977) 671.
20 N. E. Hoffman and J. C. Liao, *Anal. Chem.*, 49 (1977) 2231.
21 W. Voelter, K. Zech, P. Arnold and G. Ludwig, *J. Chromatogr.*, 199 (1980) 345.

22 C. F. Gelijkens and A. P. de Leenheer, *J. Chromatogr.*, 194 (1980) 305.
23 N. D. Brown, J. A. Kintzios and S. E. Koetitz, *J. Chromatogr.*, 177 (1979) 170.
24 A. L. Lehninger, *Biochemistry, The Molecular Basis of Cell Structure and Function*, Worth, New York, 1972, p. 637.
25 J. B. Wijngaarden and W. N. Kelly, in J. B. Stanbury, J. B. Wijngaarden and D. S. Frederickson (Editors), *Gout, The Metabolic Basis of Inherited Disease*, McGraw-Hill, New York, 1972, p. 911.
26 E. R. Giblett, J. E. Anderson, F. Cohen, B. Pollara and H. J. Meuwissen, *Lancet*, ii (1972) 1067.
27 E. R. Giblett, A. J. Ammann, R. Sandman, D. W. Wara and L. K. Diamond, *Lancet*, i (1975) 1010.