Journal of Chromatography, 203 (1981) 85–92
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,218

ZWITTERION-PAIR CHROMATOGRAPHY OF NUCLEOTIDES AND RELATED SPECIES

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SUMMARY

A new form of ion-pair chromatography is established and characterised, in which a zwitterion-pairing agent (11-amino undecanoic acid, C11AA, at up to 2 m*M* concentration in eluent) is used to enhance the retention of nucleotides in reversed-phase high-performance liquid chromatography. Excellent separations of these compounds (giving up to 5000 plates for a 100-mm column) are obtained within the pH range 4 to 6 (maintained by 75 m*M* phosphate buffer), with retention at pH 4 being up to twenty times that at pH 6. At fixed pH retention increases directly with the amount of C11AA adsorbed by the stationary phase. It is proposed that the enhancement of retention by addition of C11AA and its pH dependence arises from the formation of quadrupolar ion pairs between C11AA and the nucleotides.

The order of elution, in general, is monophosphates (least retained), diphosphates, triphosphates. However, these groups can either be separated entirely from each other, or made to interleave by suitable adjustment of pH and C11AA concentration. Deoxy- and cyclic-nucleotides are more strongly retained than their parent compounds.

The method is flexible and has great potential for further development.

INTRODUCTION

The term zwitterion-pair chromatography is used to describe reversed-phase liquid chromatography in which a zwitterionic pairing agent or hetaeron is added to the predominantly aqueous eluent. It is envisaged that such an agent will preferentially form quadrupolar ion pairs with zwitterionic solutes according to

$$S^{\pm}_{\mp} + P^{-}_{+} \rightleftharpoons \{S^{\pm}_{\mp}\ {}^{-}_{+}P\} \quad (1)$$

Aqueous phase Organic phase

However, other possible pairing reactions are not ruled out with single or multi polar species.

$$S^{+} + C^{-} + P^{-}_{+} \rightleftharpoons \{S^{\pm}P^{\pm}C\} \quad (2)$$

Aqueous phase Organic phase

$$S^{\pm}_{\mp} + B^{+} + P^{-}_{+} \rightleftharpoons \{B^{\pm}_{\mp}\ {}^{-}_{+}S^{\pm}_{\mp}P\} \quad (3)$$

Aqueous phase Organic phase

0021-9673/81/0000–0000/$02.50

The original proposal for zwitterion-pair chromatography arose out of work by us on the liquid chromatography of tetracyclines in the presence of EDTA[1] when it it was found that maximal retention of the tetracyclines occurred at a pH which was intermediate between the p*I* values (*i.e.* the value of pH at the isoelectric point) of the two components. Since the technique enabled high efficiency separations of the tetracyclines to achieved at relatively high pH (previously it had been necessary to use pH < 2) it was felt that the technique might usefully be extended to other species which could form zwitterions.

Because of their great biological importance coupled with their wide range of molecular charge, we chose nucleo-bases, nucleosides and nucleotides as model substances along with 11-amino undecanoic acid (C11AA) as the pairing agent. Current methods for the high-performance liquid chromatography (HPLC) of nucleosides and nucleotides rely mainly upon ion-exchange systems[2–11] and generally require gradient elution, although a few compounds of the group have been separated on reversed-phase systems using phosphate buffers[12–14] and cationic detergents[15,16]. There thus remains a need for a general, flexible and efficient method for the HPLC of compounds of this group.

TABLE I

pK_a AND CHARGE ON NUCLEOTIDES, NUCLEOSIDES AND BASES

Date from Albert[17] and Jordan[18]. The charges on the predominant species are indicated between the pK_a values, for example: * neutral molecule, $\pm$ zwitterion, $\underline{\pm}$ zwitterion with two negative and one positive charge.

Compound	*Primary phosphate ionization*	*Protonation*	*Secondary phosphate ionization*	*NH and OH ionization*
Adenine	+	4.3	*	9.8 −
Adenosine	+	3.5	*	12.5 −
AMP	+ 2 $\pm$	3.7 −	6.3 =	12.3 $\underline{=}$
ADP	+ 2 $\pm$ 2 $\pm$	3.9 =	6.3 $\underline{=}$	12.3 $\underline{=}$
ATP	+ 2 $\pm$ 2 $\pm$ 2 $\underline{-}\pm$	4.0 $\underline{=}$	6.5 $\underline{=}$	12.3 $\underline{=}$ =
Guanine	+	3.0	*	9.3 − 12.6 =
Guanosine	+	1.6	*	9.3 − 12.3 $\underline{=}$
GMP*	+ 2 $\pm$	2.4 − to 3.3	6.3 =	9.5 $\underline{=}$ 12.5 $\underline{=}$
Hypoxanthine	+	2.0	*	8.9 − 12.1 =
Inosine	+	1.2	*	8.8 − 12.3 =
IMP*	+ 2 $\pm$	1.5 −	6.0 =	8.9 $\underline{=}$ 12.5 $\underline{=}$
Cytosine	+	4.5	*	12.2 −
Cytidine	+	4.2	*	12.3 −
CMP*	+ 2 $\pm$	4.5 −	6.3 =	12.3 $\underline{=}$
3′5′ cyclic AMP	+ 2 $\pm$	3.7	−	12.3 =
Nicotinamide Adenine diphosphate NADH	+ 2 $\pm$ 2 $\underline{\pm}$	4.3	=	9.8 $\underline{=}$

* The ionization of the higher phosphates is analogous to that of AMP, ADP, ATP.

Vital to the discussion of any form of ion-pair chromatography is a knowledge of the charge distribution on the solutes and hetaeron to be used. Table I lists the pK_a values for the different ionization stages of a number of nucleo-bases, nucleosides and nucleotides. The data are taken from Albert[17] and Jordan[18]. The bases and nucleosides are predominantly neutral between pH 5 and 9 and have virtually no zwitterionic character. The phosphates (mono-, di- and tri-) are predominantly negatively charged because of the low pK_a for the primary phosphate groups. However, below pH 2 to 4 (depending upon the nature of the base) the phosphates, like the bases and nucleosides, possess cationic N-atoms. The secondary phosphate in all cases has pK_a in the range 6 to 6.5.

The present study covers the pH range 3.5 to 6. At the lower end of this range the compounds have cationic nitrogens, but at the upper end this ionization is suppressed. The phosphates in addition have one to three negative charges depending upon the number of primary phosphate groups present. C11AA was used throughout as hetaeron and we have assumed it to have the same pK_a values as 6-amino hexoic acid, *viz.* 4.4 (ionization of $-COOH$) and 10.4 (proton loss by $-NH^+$). C11AA therefore exists predominantly as zwitterion in the range pH 4.4 to 10.4, but, of course, there will still be a proportion of zwitterion outside these limits and significant overlap of the zwitterion ranges of the nucleotides and C11AA exists.

The study has established:

(a) that excellent separations of nucleotides can now be achieved under isocratic conditions using C11AA as hetaeron in a reversed-phase system in the pH range 4 to 6.

(b) that enhanced retention is genuinely due to the presence of adsorbed C11AA.

(c) that the degree of retention, k', and selectivity for mono-, di- and tri-phosphate nucleotides is strongly pH dependent, k' increasing as pH is reduced from 6 to 4.

(d) that the behaviour of nucleotides in the presence of adsorbed C11AA is quite different from that in the presence of 1,10-diaminodecane (10DA) at the same surface concentration.

It is proposed that these effects are attributable to the formation of quadrupolar ion pairs between the zwitterionic forms of C11AA and of the nucleotides.

EXPERIMENTAL

A Shandon UV photometric detector–oven unit (Shandon Southern Products, Runcorn, Great Britain) and an Altex Model 110 pump (Altex, Berkeley, CA, U.S.A.) formed the basic HPLC equipment. Columns were 100 mm × 5 mm I.D. Shandon pattern. Samples were injected by a Rheodyne Model 7120 valve (Shandon Southern Products). All measurements were made at 25°C.

For determination of adsorption isotherms for C11AA, an Optilab (Vallingby, Sweden) Multiref 902 refractive index monitor was employed in conjunction with an Orlita (Giessen, G.F.R.) type DMP 1515 microdosing pump. The whole of this equipment subsequent to the pump was carefully thermostatted at 25°C by circulating water.

Columns were slurry packed at 6000 p.s.i. (isopropanol) with ODS-Hypersil (Shandon Southern Products).

Water–methanol (88:12, v/v) was used as the basic eluent. The pH was adjusted by 75 m*M* phosphate buffer (KH_2PO_4 + Na_2HPO_4 with H_2PO_4 for pH below 4.6). C11AA was used as zwitterion-pairing agent at up to 2 m*M* and C10DA was used as a reference cationic pairing agent up to 6 m*M*.

Methanol was HPLC grade (Rathburn, Walkerburn, Great Britain), phosphates were AnalaR grade (BDH, Poole, Great Britain), nucleo-bases and derivatives were obtained from BDH)and from Sigma (Poole, Great Britain). C11AA and C10DA were obtained from Aldrich (Gillingham, Great Britain).

RESULTS AND DISCUSSION

Fig. 1 illustrates the excellent separation of nucleotides (3000–5000 plates) which can be achieved in the presence of C11AA. It shows the striking effect of a small change of pH upon both the degree of retention and the selectivity of the separation. This is fully detailed in Fig. 2 which plots the dependence of k' at fixed eluent concentration of C11AA (1.25 m*M*) upon pH. k' increases ten to twenty times as the pH is reduced from 6 to 4. Retention shows a maximum for some solutes at a pH of around 4.2. For dAMP there appears to be a maximum of k' at pH around 5. Maxima in k' *versus* pH curves appear to be characteristic of zwitterion-pair chromatography but data to date are insufficient to characterise this phenomenon fully. Fig. 2 also shows the variation of the concentration of adsorbed C11AA as a function of pH. It is seen that C_{ads} varies over a small range from about 70 to about 90 μmol/g, with a weak maximum at pH of 4.7. This maximum was not, however, observed with lower eluent concentration of C11AA, but rather a gradual rise of C_{ads} as pH is reduced. It

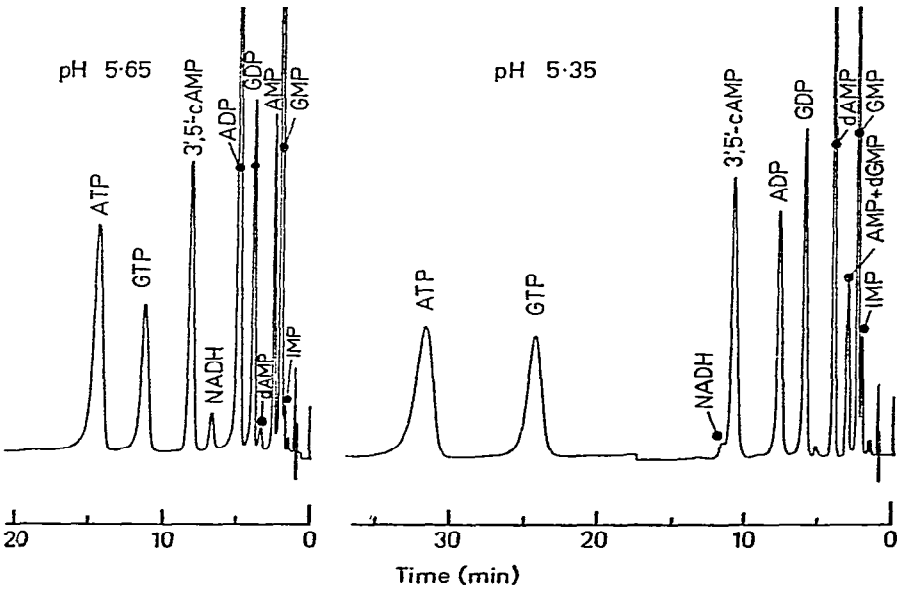

Fig. 1. Separation of nucleotides by zwitterion-pair chromatography showing the effect of pH change. Packing material: ODS-Hypersil. Eluent: water–methanol (88:12, v/v) made 75 m*M* in phosphate and 1.25 m*M* in 11-amino undecanoic acid (C11AA).

is clear that the strong dependence of k' upon pH is not accounted for by changes in the amount of C11AA adsorbed but must be related to changes in the nature of the interaction between the C11AA and the nucleotides over this range. Experiments with C10DA as pairing agent showed that C_{ads} was independent of pH over the range 4 to 6, but in striking contrast to the situation with C10AA the retention of the nucleotides was little affected by pH with no clear trends. We regard this difference as *decisive evidence for the existence of a new mode of retention when a zwitterion-pairing agent is present.*

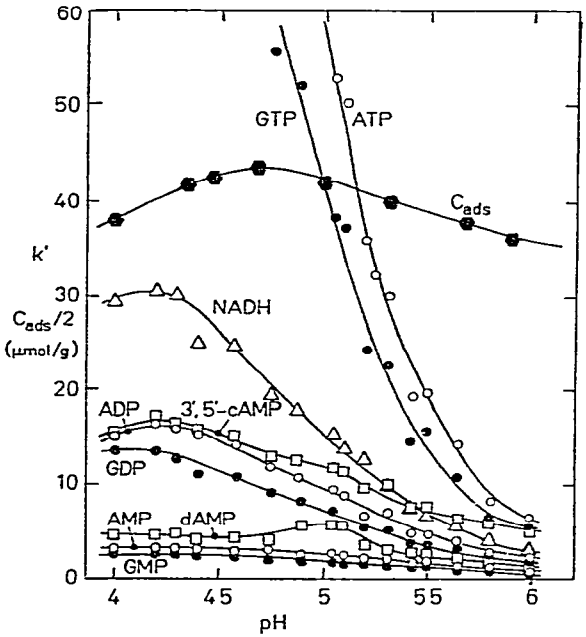

Fig. 2. Dependence of k' upon pH for zwitterion pair chromatography of nucleotides. Conditions as for Fig. 1. C_{ads} = concentration of C11AA adsorbed per gram of packing material.

Fig. 3 shows that, with one exception, k' values for nucleotides at a fixed pH (5.8) increase regularly with the concentration of C11AA in the eluent. Likewise the concentration of adsorbed C11AA increases with eluent concentration. The isotherm is linear up to an eluent concentration of about 1.5 mM, but thereafter shows some evidence of saturation. The maximum surface concentration observed was about 110 μmol/g of packing material. This corresponds to about 1 μmol/m^2 of surface area (BET measurement of surface area of ODS-Hypersil[19] yielded a value of 105 m^2/g) which is around a quarter of the concentration of bonded ODS groups as measured by carbon analysis of $\approx$8% (w/w).

The initial variation of k' with both eluent and surface concentration of C11AA is strong evidence that the additional retention caused by the amino acid is genuinely due to the formation of adducts of the amino acid and the nucleotides, as it is in simple ion-pair chromatography. Again there is a direct correlation between retention and surface concentration (see refs. 19 and 20).

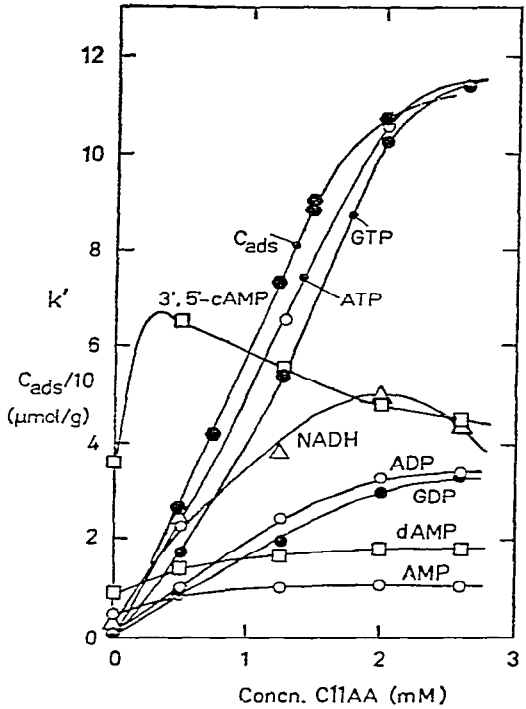

Fig. 3. Dependence of k' upon concentration of C11AA in eluent. Phosphate buffer 75 mM pH 5.8. Other conditions as Fig. 1.

It is now relevant to discuss the dependence of k' upon pH and eluent concentration in more detail.

The initial evidence from Figs. 2 and 3 implies (a) that the addition of C11AA exhances retention of nucleotides by the formation of adducts and (b) that the strength of the interaction of the average molecule of nucleotide with the average molecule of C11AA is strongly dependent upon pH. From (b) it would be natural to deduce that the pH dependence arises from the changing proportions of the forms of the nucleotide and C11AA which are responsible for the strongest interactions.

Broadly, we believe that the interactions can only be quadrupolar interactions between the C11AA zwitterions and two oppositely charged centres on the nucleotide molecule. It is, however, characteristic of the separations in the presence of C11AA that the triphosphates are most retained while the monophosphates are least retained. Inosine phosphates are less retained than guanosine phosphates and these less than adenosine phosphates. Table I indicates that the proportion of these phosphates bearing a positively charged nitrogen at any pH will also increase in the same order. The reduced phosphates (dAMP and dGMA) are more retained than the unreduced forms probably due to their lower water solubility (the former having one less OH group on the ribose ring). Similarly, cyclic AMP (with two less OH groups than AMP but otherwise similar pK_a values) is still more retained. NADH shows a behaviour intermediate between a single nucleotide diphosphate and triphosphate nucleotide.

The increasing retention with the number of phosphate groups suggests that the additional negatively charged centres introduced by addition of each phosphate group become substantially more lipophilic when paired with the amino group of C11AA, which is perhaps not an unreasonable hypothesis.

Examining Fig. 3 in more detail, it is noted that with the triphosphates ATP and GTP, proportionality of k' to C_{ads} is maintained to a high concentration of C11AA; indeed the curves for k' are slightly concave to the k'-axis. The curves for the mono- and diphosphates are by contrast convex to the k'-axis. These differences probably result mainly from the differing stoichiometry of the partitioning reactions which would predict different dependences of k' upon hetaeron concentration, [H]. Thus taking the adenosine phosphates as examples the partitioning processes and corresponding equations for k' are:

$$AMP_{(aq)} + H_{(ads)} \overset{1}{\rightleftharpoons} AMPH_{(ads)}$$

$$k'_{AMP} = \varphi K_1 [H_{ads}]$$

$$ADP_{(aq)} + 2H_{(ads)} \overset{2}{\rightleftharpoons} ADPH_{2(ads)}$$

$$k'_{ADP} = \varphi K_2 [H_{ads}]^2$$

$$ATP_{(aq)} + 3H_{(ads)} \overset{3}{\rightleftharpoons} ATPH_{2(ads)}$$

$$k'_{ATP} = \varphi K_3 [H_{ads}]^3$$

where φ is the phase ratio and K_1, K_2, K_3 are the equilibrium constants of the three reactions. While these equations do not fit the data quantitatively they explain qualitatively the changing curvature of the curves for the mono-, di- and triphosphates. The behaviour of cyclic-AMP as the concentration of C11AA changes has no obvious explanation.

Fig. 3 also shows how the order of elution and selectivity of the separation can be much altered by adjusting the C11AA concentration. Thus at very low concentrations of C11AA the order of elution of say the adenosine phosphates is ATP, ADP, AMP whereas at high concentrations the order is reversed. At concentrations of around 0.5 mM the elution order is complex: mono-, di-, and triphosphates can be eluted in a closely coupled group. At high concentrations, around 2 mM, the monophosphates, diphosphates and triphosphates elute as separate groups with no overlap apart from the rogue compound cyclic-AMP.

Under similar conditions the retention of the nucleosides, nucleobases and amines (*e.g.* benzylamine) declines with increase in concentration of C11AA whereas the retention of acids in general (for example benzoic acid, benzene sulphonic acid) increase towards maximum values. The retention of amino acids and small peptides shows intermediate behaviour with a tendency for maximum retention at an eluent concentration or around 1 mM.

CONCLUSIONS

The work has demonstrated that high efficiency separations of nucleotides can be achieved using aqueous methanol as eluent containing 11-aminodecanoic acid (up

to 2 mM) and phosphate (up to 75 mM) to buffer the mixture at pH between 4 and 6.

The values of k' are strongly affected by pH with the strongest retention observed at pH around 4.2. In striking contrast the amount of C11AA adsorbed is little affected by pH. It is proposed that this strong effect of pH arises from the changing proportions of the zwitterionic forms of the C11AA and of the nucleotides, coupling of which to form quadrupolar ion pairs is thought to be responsible for the strong retention of the nucleotides.

k' increases with the concentration of C11AA in the eluent at fixed pH of 5.8 and the adsorption isotherm is linear up to a concentration of about 1.5 mm. The maximum concentration of adsorbed C11AA is about 1 μmol/m^2.

By varying both the concentration of C11AA and pH the order of elution of the nucleotides can be altered greatly. Thus with a pH of 6 and concentrations below about 0.5 mM the mono-, di- and triphosphates are eluted with similar retention times whereas at C11AA concentration of 1.5–2 mM they are eluted as separate groups in the order mono-, di- and triphosphates. Alternatively, with a fixed concentration of C11AA (1.25 mM) and pH 6 the three phosphates elute close together whereas at pH around 5 or below they elute as separate groups.

The method is thus one of great versatility and flexibility. It is undoubtedly capable of further development and extension to the liquid chromatography of other dipolar solutes.

ACKNOWLEDGEMENT

We gratefully acknowledge the generous support of this work by the Science Research Council.

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