Journal of Chromatography, 254 (1983) 27–34
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 15,329

LIQUID CHROMATOGRAPHY WITH CROWN ETHER-CONTAINING MOBILE PHASES

III. RETENTION OF CATECHOLAMINES AND RELATED COMPOUNDS IN REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

TERUMICHI NAKAGAWA* and AKIMASA SHIBUKAWA
Faculty of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606 (Japan)
and
TOYOZO UNO
Faculty of Pharmaceutical Sciences, Mukogawa Woman's University, 4–16 Edogawa-cho, Nishinomiya-shi, 663 (Japan)

(First received August 11th, 1982; revised manuscript received September 7th, 1982)

SUMMARY

The addition of a crown ether to the mobile phase in the reversed-phase high-performance liquid chromatography of catecholamines and related compounds had some characteristic effects on their retention behaviour. When 18-crown-6 was added to an acidic mobile phase, the capacity factors of primary amino compounds were found to be appreciably enhanced, depending on the concentration of protons and the crown ether. The association constants of 18-crown-6-catecholamine complexes could be determined from experimental values of capacity factors by an equation derived from the theoretical treatment of a chromatographic model of this particular system. The effect of hydroxy and methoxy groups in catecholamine derivatives could be explained in terms of the hydrophobicity of the compounds.

INTRODUCTION

The application of crown ethers in liquid chromatography has been evaluated for the selective separation of certain classes of inorganic and organic cations based on specific complex formation. Attention has mainly been focused on the development of crown ether-modified stationary ligands; some crown ethers or cryptands were immobilized by covalent bonding to cross-linked poly(styrene–*p*-divinylbenzene)[1] and by condensation, substitution and polymerization reactions[2,3]. Poly(crown ethers) and the corresponding monomers were also immobilized on silica, which is resistant to the high pressures in high-performance liquid chromatography (HPLC)[4–6].

On the other hand, the application of crown ether-containing solutions as

0021-9673/83/0000–0000/$03.00

mobile phases has attracted some interest for the separation of metal cations and ammonium ions. The first attempt was the optical resolution of amino acids and their esters salts through chiral recognition by a crown ether dissolved in the mobile phase[7]. The selective group separation of some biogenic amines was achieved by use of a crown ether-containing mobile phase in the reversed-phase mode[8]. The ion pairing between a crown ether-associated cation and a counter anion was also used for the separation of various anions with a common anion[2].

In previous papers, we dealt with the retention behaviour of various amino compounds (aromatic amines, amides and amino acids) in reversed-phase chromatography as a function of the concentration of protons and the crown ether in the mobile phase[9], and demonstrated the applicability of the proposed method to the analysis of β-lactam antibiotics in body fluids[10].

The aim of this study was to evaluate the effect of the complexation of crown ethers with catecholamines and related compounds on their retention on a hydrophobic stationary phase.

EXPERIMENTAL

Reagents and materials

D,L-Dopa, dopamine, D,L-noradrenaline, D,L-normetanephrine, D,L-octopamine and tyramine were obtained from Nakarai Chemicals (Kyoto, Japan) and adrenaline and D,L-synephrine from Merck (Darmstadt, G.F.R.) and Sigma (St. Louis, MO, U.S.A.), respectively. 18-Crown-6 was a product of Nippon Soda (Tokyo, Japan). Reagent-grade hydrochloric acid was used to adjust the pH of the mobile phase.

Liquid chromatography

A liquid chromatograph (Trirotar-III; Jasco, Tokyo, Japan) equipped with a UV detector (Uvidec 100-III; Jasco) was used for the measurement of capacity factors. The operating conditions are given in Table I.

The samples were dissolved in water or diluted hydrochloric acid, the minimal amount required for UV detection being used in order to retain the linearity of the chromatographic system. The capacity factors were calculated from the values of

TABLE I

HPLC CONDITIONS

Detection: UV at 220 nm. Column temperature: 40°C.

Experiment	*Mobile phase*	*Flow-rate (ml/min)*	*Stationary phase and column*
k vs. [18-crown-6]	[18-Crown-6] = 0–40 m*M*, dissolved in water (pH 2.5)	1.0	Develosil ODS 10 (20 cm × 4 mm I.D.)
k vs. pH	[18-Crown-6] = 20 m*M*, dissolved in methanol–water (1:4) (pH 2.3–5.4)	1.5	Develosil ODS 10 (20 cm × 4 mm I.D.)

$(t_r - t_0)/t_0$, where t_r is retention time of a sample solute averaged over repeated measurements at the peak of the elution curve and t_0 is that of a non-absorbed substance.

Data analysis

Non-linear least-squares fittings were carried out on a PET 2001 microcomputer specially programmed in Basic[11].

THEORETICAL

The formation of complexes of crown ethers with protonated amino compounds has been reported earlier[12,13]. Accordingly, when an amino compound is introduced into an acidic mobile phase containing a crown ether, the equilibria for protonation of the amino group and association of the ammonium cation with the crown ether are taken into account, followed by the distribution of the associated and the free species between the mobile and hydrophobic stationary phases. Fig. 1 illustrates the equilibria involved in this particular system, where S and SH are the neutral and protonated species of amino compound (*e.g.*, catecholamines in this study), C and L denote the crown ether (18-crown-6) and the hydrophobic stationary phase (ODS), respectively, and the K values are the equilibrium constants specified by the subscripts. It is postulated in this model that the complex formation has a 1:1 stoichiometry, and the non-ionized amino group does not form any complex with the crown ether. The ion-pair formation between a crown ether-associated ammonium ion and a highly hydrated (hard) anion (*e.g.*, Cl^- used as the common anion in this study) is assumed hardly to affect the retention of amino compounds[10]. According to the model, we can define the capacity factor as

$$k = \varphi \cdot \frac{[LS] + [LSH] + [LCSH]}{[S] + [SH] + [CSH]} \tag{1}$$

where φ denotes the phase ratio.

It is known that the effects of a crown ether dissolved in the mobile phase on the retention of amino compounds on a hydrophobic stationary phase may act in

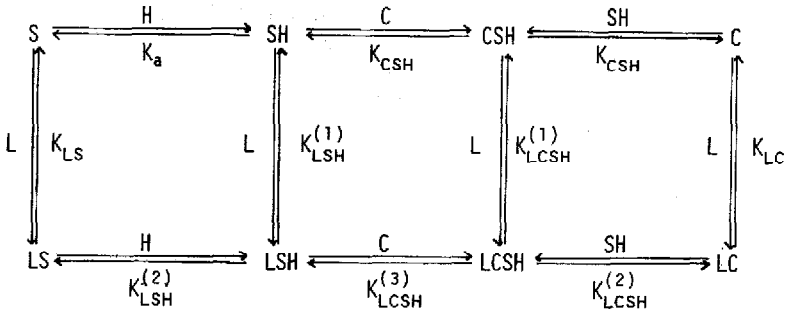


Fig. 1. Equilibria involved in reversed-phase liquid chromatography with a crown ether-containing mobile phase. C = crown ether; SH, S = protonated and neutral forms, respectively, of amine; L = hydrophobic stationary phase.

opposite directions[10], depending on the properties of the amino compounds and those of crown ether; one effect is to increase the capacity factor owing to association with amino groups and the other is to decrease it by competing with amino compounds in binding to the hydrophobic stationary phase. When the former effect is predominant over the latter, eqn. 1 can be expressed as a function of proton and crown ether concentrations in the mobile phase:

$$k = \frac{k_S K_a + (k_{SH} + k_{CSH} K_{CSH}\,[C])\,[H]}{K_a + (1 + K_{CSH}\,[C])\,[H]} \tag{2}$$

where k_S, k_{CSH}, and k_{SH} are the capacity factors of the neutral, crown ether-associated and protonated forms of the solute, respectively, which are given by $k_S = \varphi[LS]/[S] = \varphi[L_t]K_{LS}$, $k_{SH} = \varphi[LSH]/[SH] = \varphi[L_t]A$, and $k_{CSH} = \varphi[LCSH]/[CSH] = \varphi[L_t]B$, where $A = K^{(1)}_{LSH} = K_a K_{LS} K^{(2)}_{LSH}$, and $B = K^{(1)}_{LCSH} = K^{(2)}_{LCSH} K_{LC}/K_{CSH} = K^{(3)}_{LCSH} A/K_{CSH}$. Eqn. 2 predicts a rectangular hyperbolic dependence of the capacity factor on the proton concentration at a constant concentration of crown ether. For a low and constant pH, eqn. 2 can be simplified to[10]

$$k = \frac{k_{SH} + k_{CSH} K_{CSH}[C]}{1 + K_{CSH}[C]} \tag{3}$$

which also gives a rectangular hyperbolic relationship between the capacity factor and the concentration of crown ether. With dopa, which is an α-amino acid, the dependence of the capacity factor on the concentration of crown ether can also be expressed by eqn. 3, where the pH of the mobile phase is constant and low enough to prevent the dissociation of the proton of the carboxyl group[9].

RESULTS AND DISCUSSION

Effect of crown ether concentration

The effects of crown ether concentration on the capacity factors of catecholamines are shown in Fig. 2, where the pH of the mobile phase was maintained at 2.5. The increase in the concentration of 18-crown-6 from 0 to 40 m*M* gave rise to a marked increase in the capacity factors followed by a gradual approach to maxima in the k values of primary amines (tyramine, dopamine, noradrenaline, normetanephrine, octopamine), but no apparent change in those of secondary amines (synephrine, adrenaline). This difference between primary and secondary amines reflects the difference in the degree of their complexation with 18-crown-6. The results indicate that the protonated primary catecholamines interact strongly with 18-crown-6, resulting in specific enhancement of the capacity factors, whereas secondary amines associate weakly with the crown ether, probably because of steric effects, lower charge and weaker hydrogen bonding.

As mentioned before, addition of a crown ether to the mobile phase in reversed-phase liquid chromatography has two opposing effects on the retention of amino compounds. The increase in the capacity factor by complexation with protonated amino groups depends on the stability and hydrophobicity of the complex, and the decrease in the capacity factor by competing with the solute in binding to hydro-

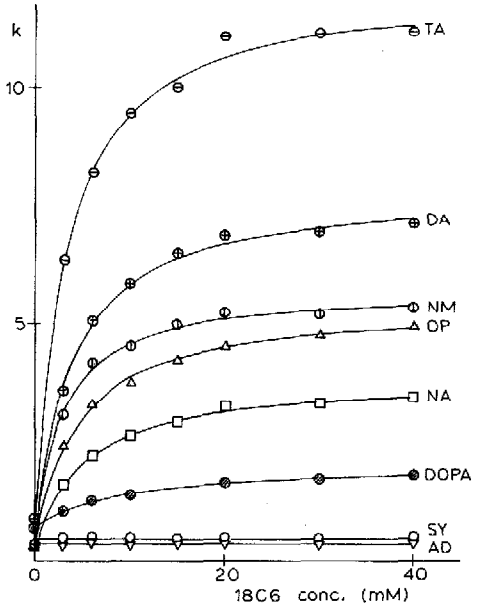


Fig. 2. Dependence of capacity factors of catecholamines and related compounds on the concentration of 18-crown-6 in the mobile phase. TA = Tyramine; DA = dopamine; NM = normetanephrine; OP = octopamine; NA = noradrenaline; SY = synephrine; AD = adrenaline.

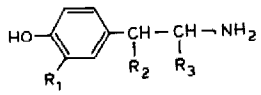
phobic stationary ligands depends on the hydrophobicity of the crown ether. Hence the overall change in the capacity factor on addition of a crown ether depends on the relative strengths of these effects. For instance, the capacity factor of several β-lactam antibiotics having a primary amino group on the lateral chain increased markedly with an initial increase in the concentration of a crown ether (18-crown-6 and dicyclohexyl-18-crown-6) followed by a gradual approach to maxima, then decreased on further addition of dicyclohexyl-18-crown-6[10]. Naturally, the capacity factors of those compounds with no amino group are subject solely to the latter effect, resulting in a greater decrease on addition of dicyclohexyl-18-crown-6.

In this study, in which 18-crown-6 was used, the change in the capacity factors of primary catecholamines as a function of 18-crown-6 concentration (Fig. 2) indicates, as expected, similar profiles to those found with β-lactam antibiotics having amino groups.

The non-linear least-squares fittings of eqn. 3 to the datapoints shown in Fig. 2 gave the k_{SH}, k_{CSH} and K_{CSH} values listed in Table II, where K_{CSH} is the stability constant for crown ether–catecholamine association in the mobile phase, k_{SH} is the capacity factor in the absence of crown ether (intercept on the ordinate in Fig. 2) and k_{CSH} is the capacity factor for a maximal increase; therefore, the ratio k_{CSH}/k_{SH} indicates the degree of enhancement of the capacity factor. The curves calculated by using these parameters are also shown in Fig. 2, indicating satisfactory agreement with the experimental values. A comparison of the results obtained for dopamine, noradrenaline, tyramine and octopamine indicates that the substitution of a hydroxy group at the β-position on the lateral chain (R_2 in Table II) causes the k_{CSH} value to

TABLE II

RELATIONSHIP BETWEEN STRUCTURE AND PERTINENT VALUES PARAMETERS IN EQN. 3 FITTED TO DATA POINTS IN FIG. 2



Compound	R_1	R_2	R_3	k_{SH}	k_{CSH}	k_{CSH}/k_{SH}	$K_{CSH}(M^{-1})$
Normetanephrine (NM)	OCH_3	OH	H	0.461	5.80	12.6	347
Tyramine (TA)	H	H	H	0.895	12.3	13.7	305
Dopamine (DA)	OH	H	H	0.504	7.89	15.7	263
Octopamine (OP)	H	OH	H	0.348	5.49	15.8	215
Noradrenaline (NA)	OH	OH	H	0.242	3.90	16.1	198
Dopa	OH	H	COOH	0.680	2.07	3.04	112

decerease to almost half of that of the unsubstituted compound, whereas on substitution of a hydroxy group at the *m*-position on the phenyl ring the value decreases to about two thirds. The substitution of a methoxy group at the *m*-position has hardly any effect. Similar trends were observed with respect to k_{SH} values. The capacity factor of dopa exhibited different behaviour, as expected from the previous results obtained for amino acids. The addition of a crown ether produced only a small increase in the capacity factor, possibly because the association of the amino group of dopa with the crown ether is restricted by the presence of an α-carboxyl group, which is consistent with the fact that the K_{CSH} value of dopa is about half of that of dopamine. These results indicate that the effect of substitution involves a change in the hydrophobicity of catecholamine itself and also in the stability of the complex with the crown ether.

Effect of pH

Fig. 3 shows the pH dependence of capacity factors in the pH range 2.3–5.4, the concentration of 18-crown-6 being kept at 20 m*M* in water–methanol (4:1). The capacity factors of catecholamines with a primary amino group exhibit, as expected, a rectangular hyperbolic decrease with increasing proton concentration, whereas the compounds bearing a secondary amino group, such as adrenaline and synephrine, show less dependence and dopa is almost independent of pH changes. Similar *k versus* $[H^+]$ profiles were also obtained when the mobile phase contained no crown ether (*i.e.*, the usual reversed-phase mode[14]), although the magnitudes of the capacity factors were then far smaller. These results indicate that as the proton concentration increases with a constant concentration of 18-crown-6, the increase in the *k* values of primary catecholamines due to complexation with 18-crown-6 is exceeded by the decrease due to an increasing concentration of free ammonium ion. This means that the ion exclusion effect at low pH predominates over the complexation effect on the retention of catecholamines on a hydrophobic stationary ligand.

The elution profiles of catecholamines obtained by using mobile phases with and without a crown ether are compared in Fig. 4. It can be seen that the addition of 18-crown-6 resolves the crowded peaks to yield a complete separation of AD, dopa, NA, OP, NM and DA, which are eluted in this order within 7 min. Naturally, this

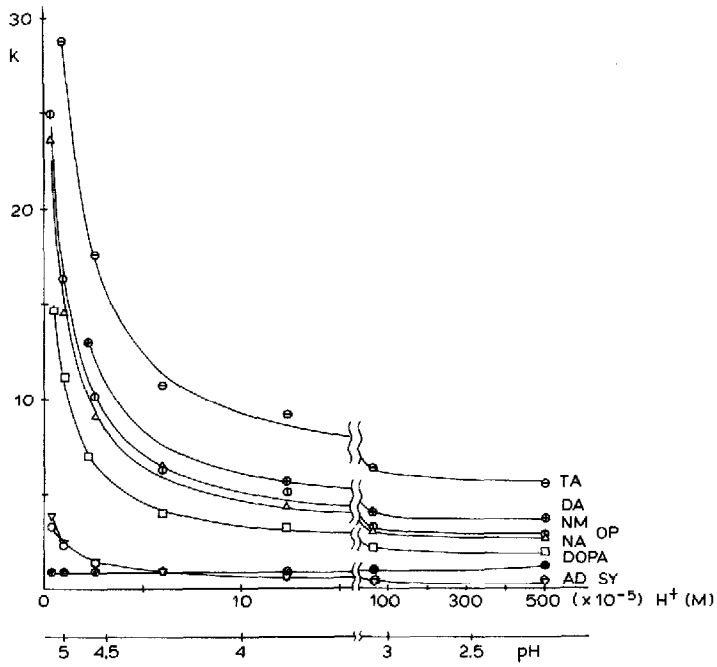


Fig. 3. Dependence of capacity factors of catecholamines and related compounds on pH of mobile phase. Abbreviations as in Fig. 2.

sequence is partly different from that observed in the usual reversed-phase mode (see Table II and Fig. 4b) and also from that in the ion-pair reversed-phase mode[15,16]. The association of ammonium ion with a hydrophobic counter ion in ion-pair chromatography is less specific to the primary amino group than that with a crown ether in the present system, because of the difference in their retention mechanism. Therefore, the retention of adrenaline (secondary amine) in ion-pair chromatography is also increased, so that it is eluted with an elapsed time between OP and NM[15], while the order of elution of primary catecholamines is same as that in Fig. 4. The complete separation of DA and OP (*para*-isomer) was not achieved by Weichmann[8], who used 0.01 *N* hydrochloric acid containing about 19 m*M* 18-crown-6 as the mobile phase. The reduction in the plate number due to deterioration of the performance of the stationary phase, which sometimes occurs when the mobile phase contains a high concentration of components such as buffer salts and ion-pairing reagents, was not appreciable in the present method. The role of inorganic salts and organic solvents in the mobile phase of this system is a problem for future investigation.

In this series of papers, we have described fundamental investigations and the practical applicability of crown ether-containing mobile phases to the HPLC of various amino compounds. The results presented here will also aid the separation of biogenic amines from biological sources, although we have not referred to the effects of crown ether on their detection, which will be considered elsewhere.

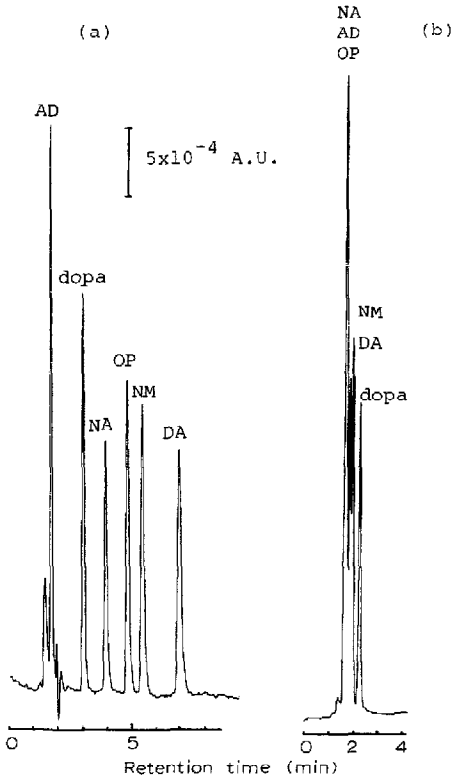


Fig. 4. Chromatograms of a standard mixture of catecholamines. Mobile phase: (a) water–methanol (9:1) containing 10 m*M* 18-crown-6 (pH 2.4); (b) water–methanol (9:1) (pH 2.4). Flow-rate: 1.0 ml/min. Stationary phase: Chemcosorb ODS-H, 5 μm (15 cm × 4.6 mm I.D.). Temperature: 40°C. Detection: UV at 220 nm. The mixture contained 5–11 ng of each catecholamine. Abbreviations as in Fig. 2.

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