CHROM. 17,446

SELECTIVE RETENTION OF CATECHOLAMINES AND THEIR DERIVATIVES IN REVERSED-PHASE ION-PAIR PARTITION CHROMATOGRAPHY

J. DE JONG, C. F. M. VAN VALKENBURG and U. R. TJADEN*

Center for Bio-Pharmaceutical Sciences, Division of Analytical Chemistry, Gorlaeus Laboratories, University of Leiden, P.O. Box 9502, 2300 RA Leiden (The Netherlands)

(Received November 14th, 1984)

SUMMARY

Some possibilities are described to improve the separation between catecholamines and their corresponding metanephrines in reversed-phase ion-pair partition chromatography. The retention of amines can be influenced by the perchlorate concentration of the mobile phase, while the retention of acidic metabolites is governed by the pH of the mobile phase. Nevertheless, a separation between the catecholamines and their corresponding metanephrines cannot be achieved by optimizing these mobile phase parameters. A decrease in the temperature of the chromatographic system leads to an improvement in the separation between dopamine and 3-methoxytyramine; however, adrenaline and noradrenaline are not separated from metanephrine and normetanephrine, respectively, and the chromatographic efficiency decreases. Addition of boric acid to the mobile phase results in a selective decrease in the retention of compounds with a catechol function, the magnitude of this effect depending on the mobile phase pH. Applying this "boric acid effect" it is possible to obtain a complete and rapid separation (within about 9 min) of the catecholamines, their corresponding metanephrines, the acidic metabolites homovanillic acid and 3,4-dihydroxyphenylacetic acid and the neutral metabolite 3-methoxy-4-hydroxyphenylethylene glycol.

INTRODUCTION

High-performance liquid chromatography (HPLC) has become increasingly important for the analysis of catecholamines and their derivatives. This is clearly illustrated by the number of papers about this subject which have been recently reviewed[1-3].

The most popular chromatographic systems, *i.e.*, reversed-phase chromatography (RPC)[4] and reversed-phase ion-pair chromatography (RPIPC)[5,6], have some drawbacks: in RP systems the retention of some compounds, *e.g.*, adrenaline, noradrenaline and 3,4-dihydroxyphenylalanine, is small, resulting in overlap with matrix peaks when biological samples are assayed[7,8]. In RPIP or so-called "soap" sys-

tems[5] problems are encountered with the stability of the phase system, manifested in a gradual decrease in the capacity ratios[6,9,10].

For these reasons an alternative phase system was developed[11,12], based on the use of tri-n-butyl phosphate (TBP) as the stationary phase and buffers containing perchlorate counter ions as the mobile phase. This chromatographic system has already been applied successfully to the analysis of (i) α-methyl-DOPA and its basic metabolites together with the biogenic amines in plasma, urine and brain samples[11,12], (ii) amino acids, dipeptides and hydrophilic acids[13], e.g., 5-hydroxyindoleacetic acid in urine[14,15], (iii) dopamine and its acidic metabolites in brain tissue[16], (iv) serotonin and its precursors and metabolites in brain tissue and urine[17] and (v) the biogenic amines and their derivatives, by applying gradient elution[18].

However, the reversed-phase ion-pair partition (RPIPP) chromatographic system has one major drawback: the 3-O-methylated metabolites of the catecholamines, the so-called "metanephrines", are not separated from their parent compounds. We investigated some possibilities to improve the selectivity of the system in this regard.

It is well known that chromatographic retention depends on the temperature. Generally, the relationship between the capacity ratio, k', and the temperature, T, can be described by the Van't Hoff isotherm[19]

$$\log k' = \frac{-\Delta G}{2.3\ RT} + \log \varphi = \frac{-\Delta H}{2.3\ RT} + \frac{\Delta S}{2.3\ R} + \log \varphi \tag{1}$$

where ΔG, ΔH and ΔS are respectively the changes in free energy, enthalpy and entropy taking place upon solute retention, R is the gas constant, T is the absolute temperature and φ is the phase ratio (V_{stat}/V_{mob}). From eqn. 1 it is obvious that a selective change of the retention of the catecholamines with respect to their corresponding metanephrines will only be obtained if ΔH of the metanephrines differs from ΔH of the catecholamines.

The complex formation between boric acid and diols/polyols has been the subject of extensive studies since the beginning of this century. From investigations concerning structural requirements it has become clear that, in aqueous media with low concentrations of the reactants, complexation only occurs if two hydroxy groups have a favourable orientation towards each other as in compounds with a catechol function and sugars like mannitol[20,21]. The complex formation is a reversible reaction and the equilibrium is strongly pH-dependent. This observation has been interpreted[21-23] as evidence that the tetrahydroxyborate anion, $B(OH)_4^-$, which is formed from boric acid at high pH values ($pK_a = 8.98$, ref. 24) is the complexing agent, as depicted in eqn. 2 for the complexation with a catechol:

$$B(OH)_4^- + \ \ \ \underset{K_{compl.,1}}{\rightleftharpoons} \ \ \ + 2H_2O \tag{2}$$
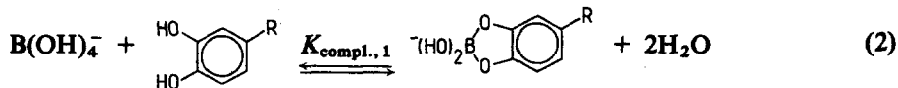
However, the same pH dependence will be observed if a complex is formed with boric acid itself:

$$B(OH)_3 + \ \ \ \underset{K'_{compl.,1}}{\rightleftharpoons} \ \ \ + H_3O^+ \tag{3}$$
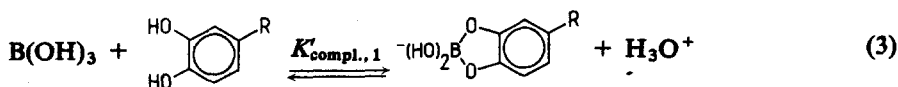
Consequently, straightforward conclusions can only be obtained from mechanistic studies[25,26]. The existence of the 1:1 complex is generally accepted. If the catechol is present in excess, a 1:2 boric acid–catechol complex may also be formed[22,27,28].

The complexation reaction between catecholamines and boric acid has also been investigated and equilibrium constants have been determined[28,29]. In some recent studies[23,30–32] the boric acid complexation has been utilized in the bioanalysis of catecholamines by means of HPLC. Boric acid itself has been applied[30], also as boric acid gels[23,32] and as diphenylborate[31]. In these reports the complex formation was only used in sample pretreatment for a selective isolation of the catecholamines. We have investigated the possibility of using boric acid to accomplish a selective change in chromatographic retention of the catecholamines.
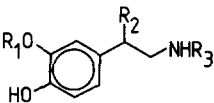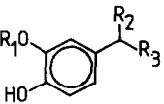
EXPERIMENTAL

Apparatus

The liquid chromatograph was constructed from custom-made and commercially available parts and consisted of a constant-flow pump (Constametric I; LDC, Riviera Beach, FL, U.S.A.), a thermostatted eluent reservoir, a Bourdon-type manometer, a Model 7120 injection system (Rheodyne, Berkeley, CA, U.S.A.) with a 20-μl sample loop, a thermostatted stainless-steel column (150 × 4.6 mm) and an amperometric detection system consisting of a so-called wall-jet detector cell unit (EDT, London, U.K.) coupled with a potentiostat (E-230; Bruker, Karlsruhe, F.R.G.). The detector cell was modified by replacement of the Ag/AgCl reference electrode by a home-made saturated calomel electrode. The potential of the glassy carbon working electrode was set at + 0.80 V *vs.* the reference electrode.

Chemicals and materials

The compounds used as chromatographic reference substances are listed in Table I. All other chemicals were of analytical or reagent grade and were used without further purification except for water, which was purified by a Milli-Q Water Purification System (Millipore, Bedford, MA, U.S.A.). The mobile phase was saturated

TABLE I

STRUCTURES OF THE CHROMATOGRAPHIC REFERENCE SUBSTANCES

Structure	R_1	R_2	R_3	Name	Abbr.	Origin
	H	OH	CH$_3$	Adrenaline	A	Boehringer, Ingelheim, F.R.G.
	H	OH	H	Noradrenaline	NA	Fluka, Buchs, Switzerland
	H	H	H	Dopamine	DA	Aldrich, Beerse, Belgium
	CH$_3$	OH	CH$_3$	Metanephrine	MN	Sigma, St. Louis, MO, U.S.A.
	CH$_3$	OH	H	Normetanephrine	NMN	Sigma
	CH$_3$	H	H	3-Methoxytyramine	3-MT	Aldrich
	CH$_3$	H	COOH	Homovanillic acid	HVA	Fluka
	H	H	COOH	3,4-Dihydroxyphenyl-acetic acid	DOPAC	Fluka
	H	OH	CHOH	3-Methoxy-4-hydroxy-phenylethylene glycol	MOPEG	Sigma

with TBP (Aldrich, Milwaukee, WI, U.S.A.) before use. Boric acid was added to the mobile phase as borax, $Na_2B_4O_7 \cdot 10 \, OH_2O$ (Brocacef, Maarssen, The Netherlands). The chromatographic support material was Nucleosil C_8, 5 μm (Macherey and Nagel, Düren, F.R.G.). Columns were packed by means of a slurry technique described elsewhere[33] and were loaded by injection of TBP until supersaturation was observed[11,12].

Chromatography

The capacity ratio of a compound was determined from its retention time and the retention time of an unretained compound, potassium iodide (10 mM).

RESULTS AND DISCUSSION

The compounds (see Table I for structures and abbreviations) can be classified as amines, acids and neutral compounds. The retention of the amines and the acids can be influenced by the counter-ion concentration (see Fig. 1) and the pH (see Fig. 2) of the mobile phase, respectively. From these plots, it can be concluded that the capacity ratio of a neutral compound (MOPEG) is not affected by these parameters. These results are in agreement with those found previously[11,12,16,18].

The separation of the nine compounds can be optimized by means of the two
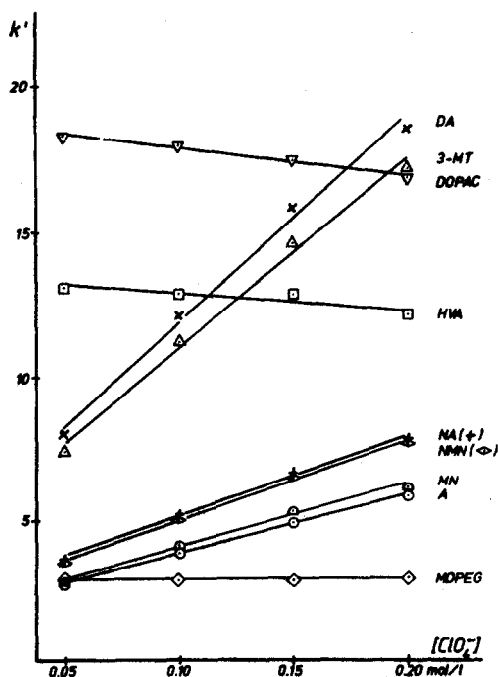

Fig. 1. Influence of the perchlorate concentration in the mobile phase on the capacity ratios (logarithmic scale) of the nine compounds (see Table I for abbreviations). Chromatographic system: stationary phase, tri-*n*-butyl phosphate coated on Nucleosil C_8, 5 μm; mobile phase, 0.05 M citrate, pH 4.9. Temperature of the phase system: 298°K.
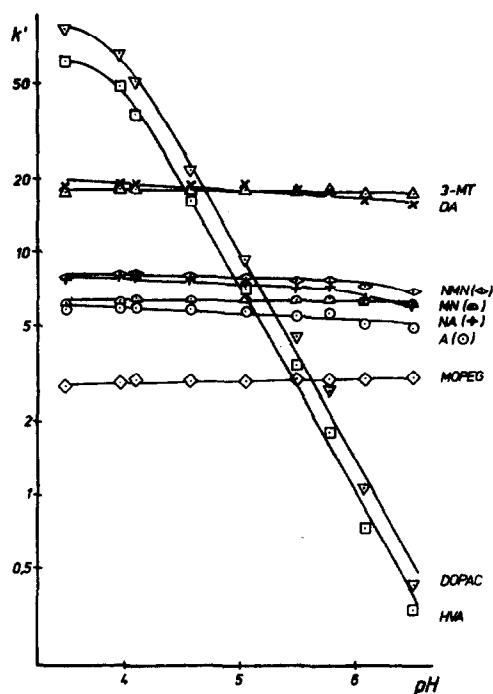
Fig. 2. Influence of the pH of the mobile phase on the capacity ratios. Perchlorate concentration: 0.20 M. For other conditions, see Fig. 1.

parameters mentioned above. Under optimized conditions (see Fig. 3), MOPEG, A, NA, HVA, DOPAC and DA are well separated from each other but the metanephrines (MN, NMN and 3-MT) are not separated from their parent catecholamines.

Effect of temperature
Until now, in the analysis of catecholamines and their derivatives by means of
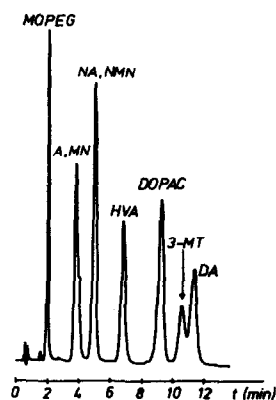

Fig. 3. Separation of the nine compounds under optimized conditions with respect to the perchlorate concentration (0.20 M) and the pH (4.9) of the mobile phase. Flow-rate: 1.8 ml/min. For other conditions, see Fig. 1.

RPIPP chromatography the column and mobile phase were thermostatted at 298°K[11,12,16,18]. Of course, it is possible to use the temperature as a third parameter to affect the retention of the compounds. Fig. 4 shows the relationship between the capacity ratios and the temperature. In agreement with theory (see eqn. 1), plots of log k' versus the reciprocal of the absolute temperature yield straight lines. The slopes of the lines for the metanephrines differ significantly from those for the corresponding catecholamines (significance level $p < 0.005$). This indicates that in the TBP–water phase system used in this study replacement of the 3-hydroxy group in the solute molecule by a 3-methoxy group leads to a change in enthalpy (see eqn. 1). The consequences for chromatographic separation are shown in Fig. 5: a decrease in the temperature leads to an improvement of the separation between 3-MT and DA and at 293°K these two compounds are almost baseline-separated. However, it is still impossible to separate A from MN and NA from NMN. Moreover, a decrease in the temperature results in an increase in the capacity ratios (see Fig. 4) and thus —despite the fact that the flow-rate is increased from 1.8 to 2.1 ml/min— the analysis time increases to about 15 min. Finally, a decrease in the temperature results in a decrease in the chromatographic efficiency. This phenomenon, which is generally observed in chromatography, is mainly caused by an increase in the contribution of
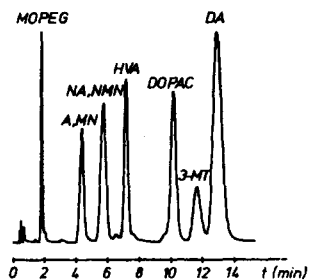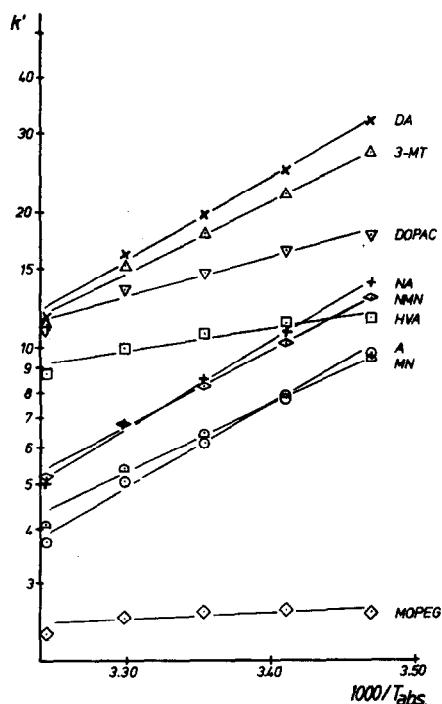

Fig. 4. Van't Hoff plot (k' on logarithmic scale) for the temperature range 288–308 °K. Perchlorate concentration; 0.20 M. For other conditions, see Fig. 1.

Fig. 5. Separation of the nine compounds at 293°K. Perchlorate concentration: 0.20 M. Flow-rate: 2.1 ml/min. For other conditions, see Fig. 1.

the "resistance to mass transfer" terms to peak broadening through a decrease in the solute diffusion coefficients[34].

Effect of boric acid in the mobile phase

The influence of the addition of boric acid to the mobile phase on the retention of compounds with a catechol function strongly depends on the pH of the mobile phase (see Fig. 6). A comparison with Fig. 2 clearly shows that at pH values of about 3.5 the presence of 0.40 M boric acid does not affect the retention of the catechol-amines. At higher pH values its influence becomes more and more pronounced and at about pH 7.0 the catechols are hardly retained. These results can be explained with the mechanism presented in Fig. 7: if the mobile phase does not contain boric acid the protonated catecholamines (DH$^+$) are retained as ion pairs with perchlorate counter ions (X$^-$)[11,12,16,18]; ion-pair extraction involves adduct formation with TBP (S)[12]. If boric acid is present in the mobile phase, at low pH the complexation between the catecholamines and boric acid as described in eqns. 2 and 3 is negligible because the concentration of B(OH)$_4^-$ (denoted as Bor$^-$) is very low and that of H$_3$O$^+$ is very high. At higher pH the complexation becomes more important. This results in a decrease in the retention because neither the boric acid–catecholamine complex (the zwitterion $^-$BorDH$^+$) nor its perchlorate ion pair (the negatively charged $^-$BorDH$^+$X$^-$) will be extracted to an appreciable extent into the organic phase. [For reasons of simplicity, only the reaction between B(OH)$_4^-$ and a catecholamine as
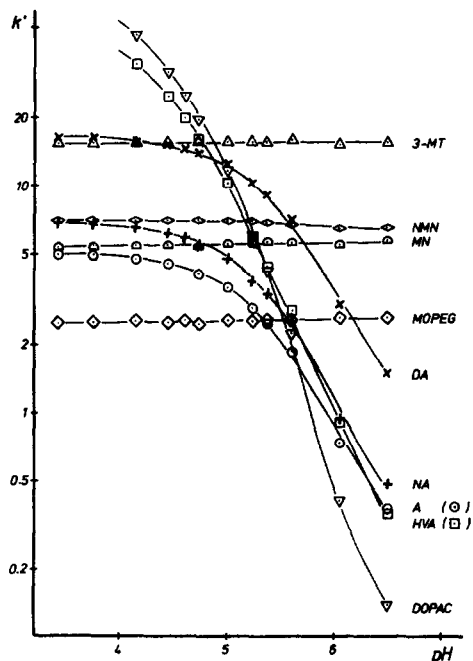

Fig. 6. Influence of the mobile phase pH on the capacity ratios (logarithmic scale) in the presence of boric acid in the mobile phase (0.10 M borax). Perchlorate concentration: 0.20 M. For other conditions, see Fig. 1.

$$DH^+X^-S_n$$

$$org. \qquad +nS$$
$$aq. \text{- -}$$

$$K_{ex} \qquad\qquad\qquad\qquad {}^-BorDH^+X^-$$

$$H_3O^+ \qquad\qquad\qquad\qquad K_{i.p.,1}$$

$$+$$

$$DH^+ \; + \; Bor^- \xrightarrow{K_{compl.,1}} {}^-BorDH^+ \; + \; 2\,H_2O$$

$$+ \qquad\qquad K_a \qquad\qquad\qquad +$$

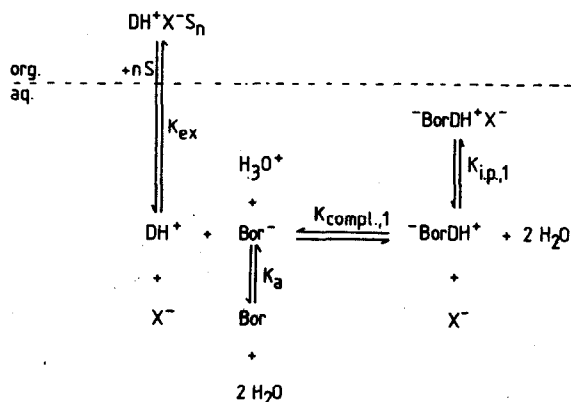$$X^- \qquad Bor \qquad\qquad\qquad X^-$$

$$+$$

$$2\,H_2O$$

Fig. 7. Influence of boric acid in the mobile phase on the retention mechanism of catecholamines.

described in eqn. 2 is depicted in Fig. 7, but is is also possible that the complex is formed from boric acid itself (denoted as Bor) as described in eqn. 3.]

In mobile phases without boric acid, acidic compounds (*viz.*, HVA and DOPAC) are only retained in their neutral forms. Thus, if at increasing pH the acids are deprotonated the retention decreases (see Fig. 2). If the mobile phase contains boric acid, for acidic compounds with a catechol function (DOPAC) there is an additional decrease in retention at higher pH because of the formation of a negatively charged complex with boric acid. Consequently, the order of elution of HVA and DOPAC is reversed at higher pH values (see Fig. 6).

In Fig. 8 the combined influence of the pH and the boric acid concentration of the mobile phase is shown. These three-dimensional plots clearly indicate that at a fixed pH an increase in the boric acid concentration leads to an increase in the retention of compounds with a catechol function, but that the extent of the "boric acid effect" is primarily determined by the pH of the mobile phase.

The separation between the nine compounds can be optimized by making use of the plots of Fig. 8. Criteria for optimization are primarily the resolution between each pair of compounds and secondly the range of k' values. The latter criterion implies that MOPEG (k' is about 2.9 at 298°K) must be the first compound eluted. Fig. 9 shows the separation of the nine compounds under optimized conditions. All compounds are almost baseline-separated and the analysis is completed in about 9 min. Compared to the system without boric acid in the mobile phase (see Fig. 3, pH = 4.9), the addition of 0.09 M borax to the mobile phase (buffered at pH 5.0) causes a selective change in retention for A, NA and DA: the elution order of 3-MT and DA is reversed, while A and NA now are also eluted prior to MN and NMN.

Formation of 2:1 complexes

The "boric acid effect" has been explained assuming that only 1:1 complexes are formed between compounds with a catechol function and boric acid (see Fig. 7). Because 2:1 complexation has only been demonstrated when the catechol is present in excess[22,27,28], this assumption seems to be justified: in the experiments described in this paper a small amount of the catechols (20 μl of a 1 mM solution) was injected
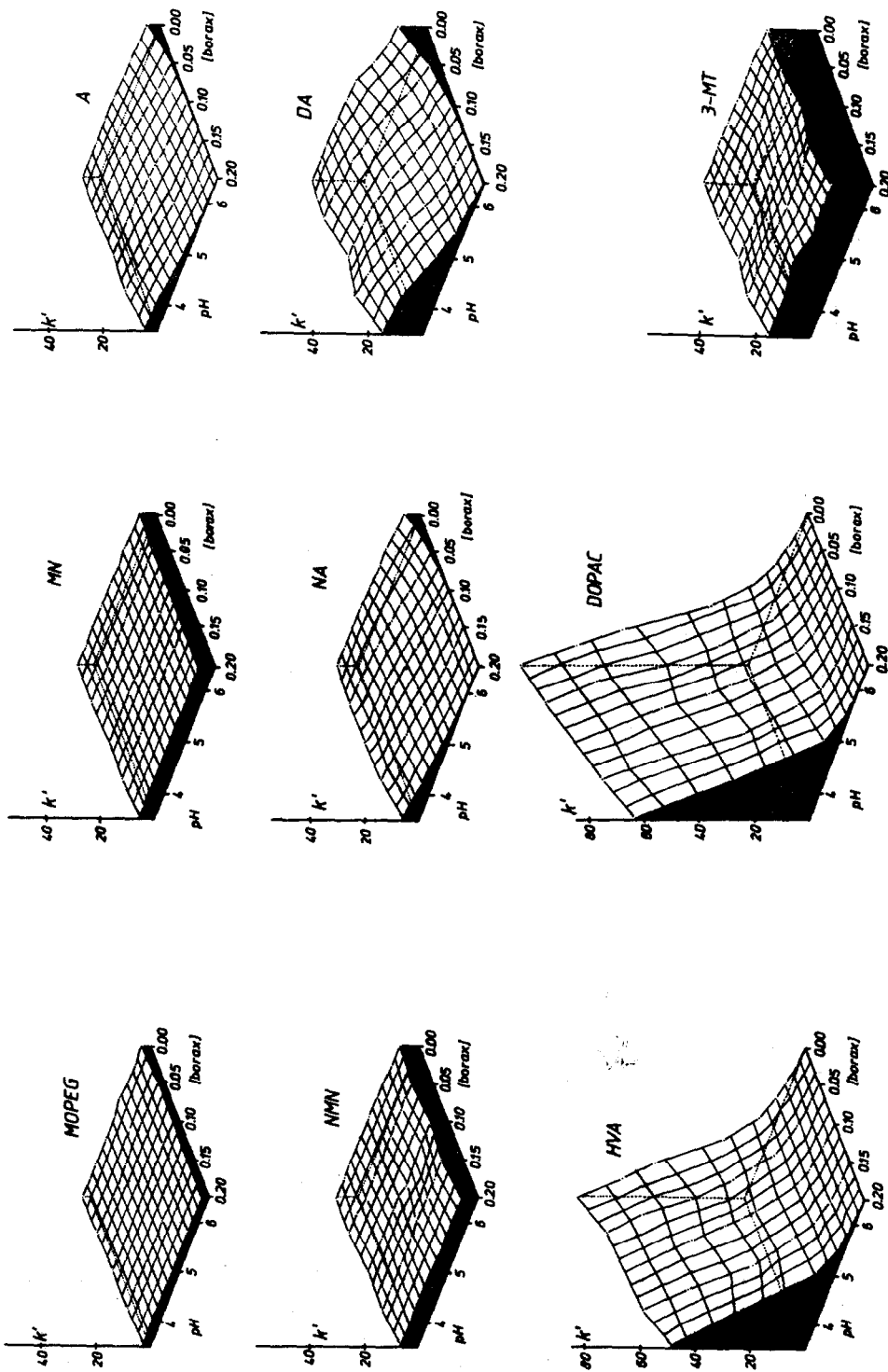
Fig. 8. Combined influence of the pH and the boric acid concentration (expressed as the concentration in mol/l of borax added to the mobile phase) on the capacity ratios. Perchlorate concentration: 0.20 M. For other conditions, see Fig. 1.
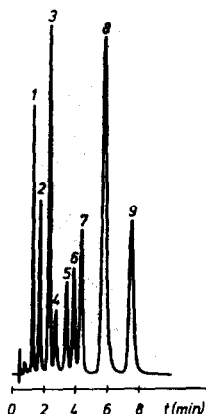
Fig. 9. Separation of the nine compounds under optimized conditions with respect to the boric acid concentration (0.09 *M* borax), the pH (5.0) and the perchlorate concentration (0.20 *M*). Flow-rate: 2.1 ml/min. Compounds: 1 = MOPEG; 2 = A; 3 = NA; 4 = MN; 5 = NMN; 6 = HVA; 7 = DOPAC; 8 = DA; 9 = 3-MT. For other conditions, see Fig. 1.

in a mobile phase containing a large excess of boric acid (0.20–0.80 *M*). At these molar ratios only the existence of the 1:1 complexes has been proven[26,28,35].

Evidence that 2:1 complexation does not play a significant rôle can be obtained from the shape of the chromatographic peaks of A, NA and DA (see Fig. 8). If 2:1 complexation were to occur, the retention mechanism depicted in Fig. 7 must be extended with at least one further equilibrium:

$$2\,DH^+ + Bor^- \overset{K_{compl.,2}}{\rightleftharpoons} {}^-Bor(DH^+)_2 + 4\,H_2O \tag{4}$$
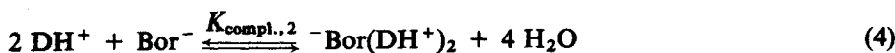
For obvious reasons the 2:1 complex $^-Bor(DH^+)_2$ will not be extracted into the organic phase and consequently for the distribution coefficient, *D*, of the catecholamines, a corresponding term must be included in the denominator:

$$D = \frac{[DH^+X^-S_n]_{org}}{[DH^+]_{aq} + [^-BorDH^+]_{aq} + [^-BorDH^+X^-]_{aq} + [^-Bor(DH^+)_2]_{aq}} \tag{5}$$

Combination of eqns. 4 and 5 and the equilibria as described in Fig. 7 results in the following equation for the distribution coefficient of a catecholamine:

$$D = \frac{K_{ex}[X^-]_{aq}[S]_{org}^n}{1 + \dfrac{K_{compl.,1}K_a[Bor]_{aq}}{[H_3O^+]_{aq}}(1 + K_{i.p.,1}[X^-]_{aq}) + \dfrac{K_{compl.,2}K_a[Bor]_{aq}[DH^+]_{aq}}{[H_3O^+]_{aq}}} \tag{6}$$

The last term in the denominator of eqn. 6, which represents the contribution of the 2:1 complex, depends on the catecholamine concentration in the aqueous phase, $[DH^+]_{aq}$, which differs in a chromatographic zone. This means that if 2:1 complexation were to occur to an appreciable extent the distribution coefficient, and thus the

retention, would be smaller at the maximum of the peak than at the front or back and consequently strongly tailing peaks would emerge. (It is not likely that other processes occurring simultaneously during chromatography, *e.g.*, longitudinal diffusion and resistance to mass transfer from one phase to the other, would mask this ·peak tailing.) The results of the chromatographic separation (see Fig. 9: very efficient separation; no significant tailing of the A, NA and DA peaks) indicate that the assumption that 2:1 complexation does not play a significant rôle is correct.

ACKNOWLEDGEMENTS

REFERENCES

1 A. M. Krstulović, *J. Chromatogr.*, 229 (1982) 1.
2 I. N. Mefford, *J. Neurosci. Methods*, 3 (1981) 207.
3 S. Allenmark, *J. Liquid Chromatogr.*, 5 (1982) 1.
4 I. Molnar and Cs. Horváth, *Clin. Chem.*, 22 (1976) 1497.
5 J. H. Knox and J. Jurand, *J. Chromatogr.*, 125 (1976) 89.
6 J. P. Crombeen, J. C. Kraak and H. Poppe, *J. Chromatogr.*, 167 (1978) 219.
7 C. Hansson, G. Agrup, H. Rorsman, A.-M. Rosengren, E. Rosengren and L.-E. Edholm, *J. Chromatogr.*, 162 (1979) 7.
8 J. Wagner, M. Palfreyman and M. Zraika, *J. Chromatogr.*, 164 (1979) 41.
9 G. Scratchley, A. N. Masoud, S. J. Stohs and D. W. Wingard, *J. Chromatogr.*, 169 (1979) 313.
10 H. J. L. Janssen, U. R. Tjaden and C. F. M. van Valkenburg, unpublished results.
11 H. J. L. Janssen, U. R. Tjaden, H. J. de Jong and K.-G. Wahlund, *J. Chromatogr.*, 202 (1980) 223.
12 H. J. L. Janssen, *Ph.D. Thesis*, University of Leiden, 1981.
13 K.-G. Wahlund and B. Edlén, *J. Liquid Chromatogr.*, 4 (1981) 309.
14 K.-G. Wahlund and B. Edlén, *J. Chromatogr.*, 204 (1981) 269.
15 K.-G. Wahlund and B. Edlén, *Clin. Chim. Acta*, 110 (1981) 71.
16 C. van Valkenburg, U. Tjaden, J. van der Krogt and B. van der Leden, *J. Neurochem.*, 39 (1982) 990.
17 J. de Jong, U. R. Tjaden, W. van 't Hof and C. F. M. van Valkenburg, *J. Chromatogr.*, 282 (1983) 443.
18 U. R. Tjaden, J. de Jong and C. F. M. van Valkenburg, *J. Liquid Chromatogr.*, 6 (1983) 2255.
19 W. Melander, D. E. Campbell and Cs. Horváth, *J. Chromatogr.*, 158 (1978) 215.
20 J. Böeseken, *Advan. Carbohyd. Chem.*, 4 (1949) 189.
21 H. Steinberg, *Organoboron Chemistry*, Vol. 1, Interscience, New York, 1964, Ch. 15.
22 K. Yoshino, M. Kotaka, M. Okamoto and H. Kakihana, *Bull. Chem. Soc. Jap.*, 52 (1979) 3005.
23 S. Higa, T. Suzuki, A. Hayashi, I. Tsuge and Y. Yamamura, *Anal. Biochem.*, 77 (1977) 18.
24 N. Ingri, *Acta Chem. Scand.*, 16 (1962) 439.
25 L. Babcock and R. Pizer, *Inorg. Chem.*, 19 (1980) 56.
26 R. Pizer and L. Babcock, *Inorg. Chem.*, 16 (1977) 1677.
27 M. Pasdeloup and C. Brisson, *Org. Magn. Reson.*, 16 (1981) 164.
28 P. J. Antikainen and K. Tevanen, *Suomen Kem.*, B 39 (1966) 285.
29 P. J. Antikainen and U. Witikainen, *Finn. Chem. Lett.*, (1976) 165.
30 R. E. Shoup and P. T. Kissinger, *Clin. Chem.*, 23 (1977) 1268.
31 F. Smedes, J. C. Kraak and H. Poppe, *J. Chromatogr.*, 231 (1982) 25.
32 C. Hansson, G. Agrup, H. Rorsman, A.-M. Rosengren and E. Rosengren, *J. Chromatogr.*, 161 (1978) 352.
33 U. R. Tjaden, M. T. H. A. Meeles, C. P. Thys and M. van der Kaay, *J. Chromatogr.*, 181 (1980) 227.
34 D. E. Henderson and D. J. O'Connor, *Advan. Chromatogr.*, 23 (1984) 65.
35 R. Meillleur and R. L. Benoit, *Can. J. Chem.*, 47 (1969) 2569.