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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF BIOGENIC AMINES

I. USE OF AQUEOUS ACIDIC MOBILE PHASES WITH SILICA COLUMNS

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SUMMARY

The separation of catecholamines and indoles using aqueous acids as mobile phases with silica columns was investigated. The effects on retention of different acids, pH variation, ionic strength and organic solvents were studied. A retention model based on a quasi-reversed-phase partition at pH $\leq$ 2, supplemented by ionic interactions with silica at pH $>$ 2, is suggested. With the aqueous acid–silica system the columns showed high efficiency, high reproducibility and extraordinary long lifetimes.

INTRODUCTION

The development of high-performance liquid chromatographic (HPLC) methods for the determination of biogenic amines and their metabolites has resulted in several approaches. In addition to procedures using ion-exchange columns[1], more recent methods are usually based on reversed-phase chromatography with hydrophobic stationary phases[2–5], often in the presence of ion-pair reagents. The advantages of reversed-phase systems over ion exchangers are improved efficiency and reproducibility at higher speed. The ion-pair systems do, however, suffer from the same basic problem as the ion exchangers, i.e., the absence of reproducible retention as a function of load. In addition, the long equilibration times and high temperature sensitivity limit the use of ion-pair systems. Without trying to reduce the great potential of reversed-phase columns, the limitations to their use should also be acknowledged. No matter how well a column is kept and protected, using pre-columns, etc., a slow but continuous 'erosion' of the bonded stationary phase takes place. The gradual appearance of more active polar sites results in peak broadening, loss of resolution and poor retention reproducibility, especially with basic solutes. A column lifetime of only a few weeks is not uncommon.

Silica is a possible alternative packing, but not when adsorption mechanisms are involved, which creates more problems with basic solutes than can be solved. Persson and Karger[6] have shown straight-phase partition in the presence of ion-pair

reagents to be one alternative. Wheals[7] made use of the ion-exchange capability of silica at pH 10, and found that the solubility of silica at high pH was no problem with large amounts of methanol in the aqueous mobile phase. The use of aqueous acids as the mobile phase with silica columns had not been studied until Crommen[8] considered it in a recent paper.

In this work the determination of biogenic amines using a silica column and aqueous acids as the mobile phase was examined, special attention being paid to the requirements for routine methods, such as high speed, high reproducibility and long column lifetimes. The results are discussed in relation to the recent work of Crommen with aqueous ion-pair reagents on silica.

EXPERIMENTAL

Apparatus

The HPLC equipment consisted of Waters Model 6000A solvent-delivery system, a Waters U6K valve-loop injector and a Waters Model 440 absorbance detector (280 nm). For the detection of alcohols a Waters R 401 differential refractometer was used.

One column (4.6 × 250 mm I.D.) was packed with 5-μm Spherisorb S5 silica (Phase Separations, Queensferry, Great Britain), which had a specific surface area of 220 m^2/g. Injection of epinephrine gave an HETP of 0.05 mm, with an asymmetry factor, $A_s = b/a$, of 2.0, measured at 10% of the peak height.

The other column (3.9 × 300 mm I.D.) was packed with 10-μm μPorasil-60A silica (Waters Assoc., Milford, MA, U.S.A.), which had a specific surface area of 500 m^2/g. The packing was deactivated (not silanized) by the manufacturer to be useful for gel filtration, but specific information on the deactivation procedure could not be obtained. With epinephrine, HETP values of 0.10 mm were obtained, with an asymmetry factor of 1.8.

The pH measurements were made with a Beckman H5 pH meter with an Ingold 405-M3 combined microelectrode or with a Metrohm E 396B pH meter.

Detection

UV detection was used throughout this work. A comparison of UV, electrochemical and fluorescence detection of catecholamines and indoles has been made and will be published separately[9].

Chemicals

Doubly distilled water was used. Dodecyl hydrogen sulphate, perchloric acid (70°C), sodium perchlorate, trichloroacetic acid, formic acid (98–100%) and sulphuric acid (95–97%) (all pro analysi grade) were obtained from Merck (Darmstadt, G.F.R.). Monochloroacetic acid (purum grade) and dichloroacetic acid (puriss grade) were supplied by Fluka (Buchs, Switzerland), acetic acid (99.5%) by Baker (Phillipsburgh, NJ, U.S.A.) (analyzed reagent grade) and methanol and acetonitrile by Rathburn (Walkerburn, Great Britain; HPLC grade). All the standard substances were obtained from Sigma (St. Louis, MO, U.S.A.).

Stock solutions of the standards in 0.01 M perchloric acid (*ca.* 1 mg/ml) were stored at −20°C. Owing to the limited stability of indoles and N-acetyldopamine, fresh

soutions were prepared every 3 weeks. The limited solubility of melatonin, N-acetyl-serotonin and methylated tryptamines was overcome by the prior addition of 25 μl of acetonitrile to the 0.01 M perchloric acid.

Chromatographic procedures

The mobile phases were degassed for 1 h in an ultrasonic bath. After storage the columns were washed with water for 20 min at 2 ml/min. After a change of mobile phase, equilibration for 10 min at 2 ml/min was sufficient, as controlled with a standard mixture of indoles. All separations were performed at room temperature. After use the silica columns were washed with water and stored in methanol.

RESULTS AND DISCUSSION

Effect of acids

In order to examine the effect of different acids, aqueous solutions of acetic acid, formic acid, monochloroacetic acid (MCA), dichloroacetic acid (DCA), trichloroacetic acid (TCA), sulphuric acid and perchloric acid were tested as mobile phases for chromatography of one group of ten catecholamines and one group of eight indoles on silica. The 0.1 M concentrations of the organic acids were chosen to ensure a large excess if any ion-pair effects were present. Decreasing the concentration of perchloric acid from 0.1 to 0.01 M had no effect on the retention, but could have a positive effect on the column stability by increasing the pH from 1 to 2, as the specific pore volume of porous silica is constant at pH 2^{10}; 0.01 M sulphuric acid gave the same results as 0.01 M perchloric acid and therefore has not been included in Tables I and II. The dissociation constants of the organic acids and the pH of the aqueous solutions are given in Table III.

The retention data and the chromatograms showed that weak acids, such as acetic acid and to some extent also formic acid, produced highly retained, broad peaks of solutes with basic functions, especially the substituted catecholamines and tryptamines. The acidic compounds had lower retentions and sharp peaks. The retention was only weakly affected by the acid strength. The neutral compound melatonin gave broad, well retained peaks in all of the media. Generally, the best efficiency and resolution were obtained with perchloric and trichloroacetic acids. The lack of resolution between EPI. DA and NMET showed that the separation system had limitations.

pH variation

In order to study the influence of pH on the retention at a constant concentration of anions, a group of catecholamines and indoles were chromatographed with perchlorate (Fig. 1) and trichloroacetate (Fig. 2) at different pH. The pH was varied by adding 6 M sodium hydroxide solution to 0.1 M solutions of the acids and measured with a pH meter. At pH > 3 the retention of NE, DA, EPI and EPI-Me increased to $k' > 20$ with both perchlorate and trichloroacetate, and the peaks became broad and asymmetric. For T, 5-HT, NMET and MET the increase in retention was significantly lower, and lower with perchlorate than trichloroacetate. For IAA, 5-HIAA, N-Ac-5-HT, MEL and HVA the retention decreased with increasing pH, especially at pH 4–5.
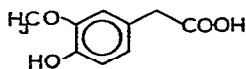
TABLE I

CAPACITY FACTORS OF CATECHOLAMINES AND HOMOVANILLIC ACID ON SPHERI-
SORB SILICA (5 μm) WITH AQUEOUS ACIDS AS THE MOBILE PHASE

Catecholamines:
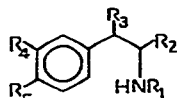
Homovanillic acid (HVA):

Compound	Abbreviation	R_1	R_2	R_3	R_4	R_5
Norepinephrine	NE	H	H	OH	OH	OH
Epinephrine	EPI	CH_3	H	OH	OH	OH
Dopamine	DA	H	H	H	OH	OH
Normetanephrine	NMET	H	H	OH	OCH_3	OH
Metanephrine	MET	CH_3	H	OH	OCH_3	OH
Epinephrine methyl ether	EPI-Me	CH_3	H	OCH_3	OH	OH
N-Acetyldopamine	N-Ac-DA	$COCH_3$	H	H	OH	OH
Dopa	DOPA	H	COOH	H	OH	OH

Compound	0.01 M HClO$_4$	0.1 M TCA	0.1 M DCA	0.1 M MCA	0.1 M HCOOH	0.1 M HOAc
NE	0.27	0.21	0.31	0.59	0.60	5.35
EPI	0.52	0.56	0.52	0.97	2.68	7.9
DA	0.52	0.56	0.52	0.97	2.68	7.9
NMET	0.52	0.56	0.52	0.97	2.84	8.0
MET	1.35	1.48	1.22	2.24	6.6	17.1
EPI-Me	1.29	1.22	1.06	1.92	6.0	16.1
HVA	0.94	0.76	0.65	0.68	0.90	0.68
N-Ac-DA	1.16	1.00	0.84	0.90	1.16	1.03
DOPA	0.05	0.21	—	0.37	—	—

Thus, different selectivities could be obtained by varying the pH. At pH 1–3 the type of anion was less important, while at pH > 3–4 more distinct effects could be obtained with trichloroacetate than with perchlorate.

Examples of changes in selectivity are shown in Figs. 3 and 4.

Ionic strength

If the retentions (k') in 0.1 M acetic acid (pH 3) in Tables I and II are compared with those in 0.1 M perchloric acid (pH 3) and 0.1 M trichloroacetic acid (pH 3) in Figs. 1 and 2, a striking difference is observed. Solutes with basic functions were retained very strongly in 0.1 M acetic acid (pH 3) whereas acidic and neutral solutes were little affected. The cation (H^+) concentration in 0.1 M acetic acid was 0.001 M, whereas the cation (Na^+) concentration in the two other solvents was close to 0.1 M.

Apparently the cation-exchange capability of silica at this pH was sufficient to

TABLE II

CAPACITY FACTORS OF INDOLES ON SPHERISORB SILICA (5 μm) WITH AQUEOUS ACIDS AS MOBILE PHASE

Indoleamines:
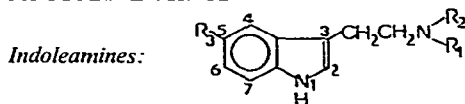
Compound	Abbreviation	R_1	R_2	R_3
Tryptamine	T	H	H	H
Serotonin	5-HT	H	H	OH
N-Acetylserotonin	N-Ac-5-HT	H	$COCH_3$	OH
Melatonin	MEL	H	$COCH_3$	OCH_3
N-Methyltryptamine	N-Me-T	H	CH_3	H
N,N-Dimethyltryptamine	N,N-Di-Me-T	CH_3	CH_3	H

Indolic acids:
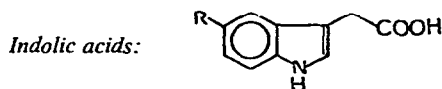
Indole-3-acetic acid (IAA), R = H
5-Hydroxyindole-3-acetic acid (5-HIAA), R = OH
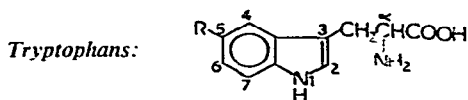
Tryptophans:

Tryptophan (TRP), R = H
5-Hydroxytryptophan (5-HTP), R = OH
5-Methyltryptophan (5-Me-TRP), R = CH_3

Compound	0.01 M $HClO_4$	0.1 M TCA	0.1 M DCA	0.1 M MCA	0.1 M HCOOH	0.1 M HOAc
5-HT	0.68	0.71	0.65	1.27	3.63	10.4
T	2.56	2.61	1.73	3.19	11.7	28
5-HTP	0.40	0.46	0.43	0.68	1.09	1.86
TRP	1.22	0.84	0.95	1.36	1.89	2.37
5-HIAA	0.45	0.37	0.33	0.33	0.40	0.33
IAA	1.06	0.78	0.65	0.70	1.15	0.80
N-Ac-5-HT	1.54	1.25	1.06	1.19	1.54	1.32
MEL	7.6	5.3	3.9	4.5	7.3	5.4

TABLE III

DISSOCIATION CONSTANTS AND pH VALUES IN AQUEOUS SOLUTIONS

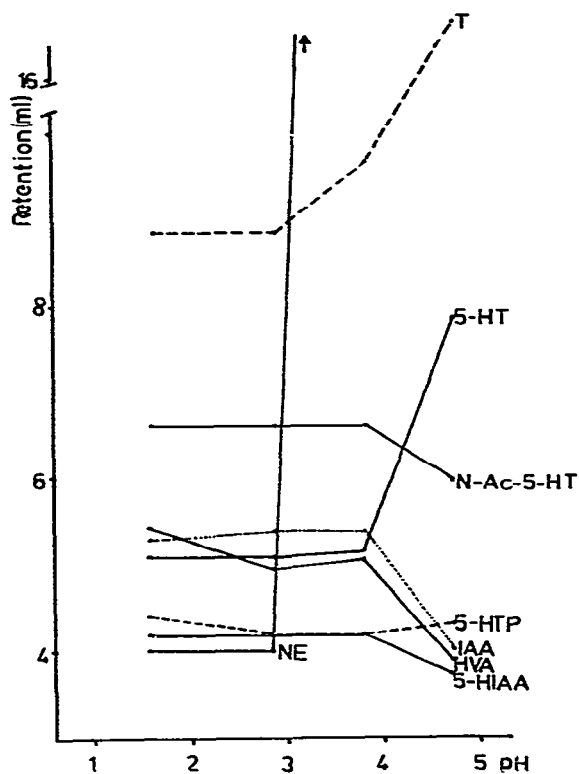| Acid	pK_a	pH in 0.1 M solution
TCA	0.70	1.3
DCA	1.48	1.5
MCA	2.85	2.1
HCOOH	3.75	2.5
HOAc	4.75	3.0

Fig. 1. Retention on Spherisorb S5 silica as a function of pH with 0.1 M HClO$_4$ + NaOH as mobile phase.
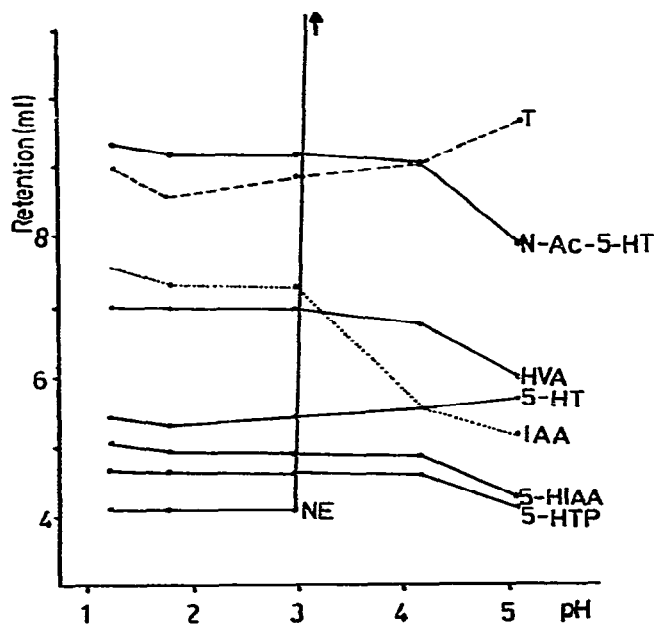

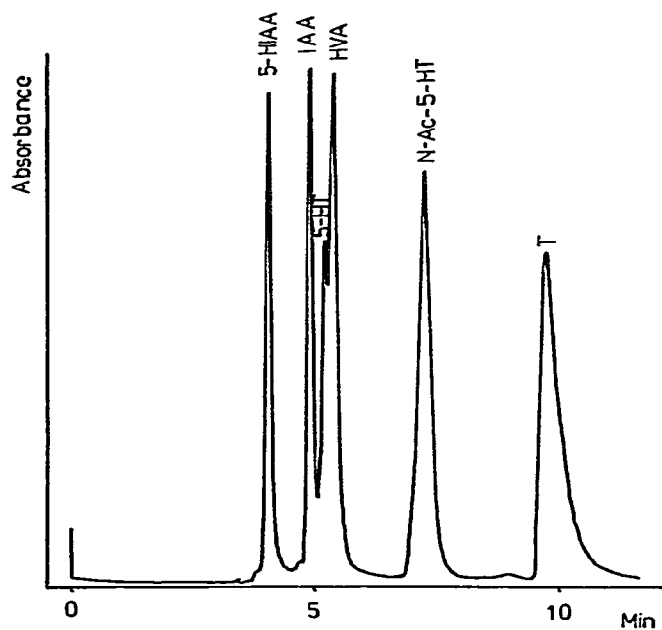Fig. 2. Retention on Spherisorb S5 silica as a function of pH with 0.1 M TCA + NaOH as mobile phase.

Fig. 3. Separation of six components involved in the metabolism of tryptophan with 0.1 M NaClO$_4$ (pH̄ 5.05) as mobile phase.
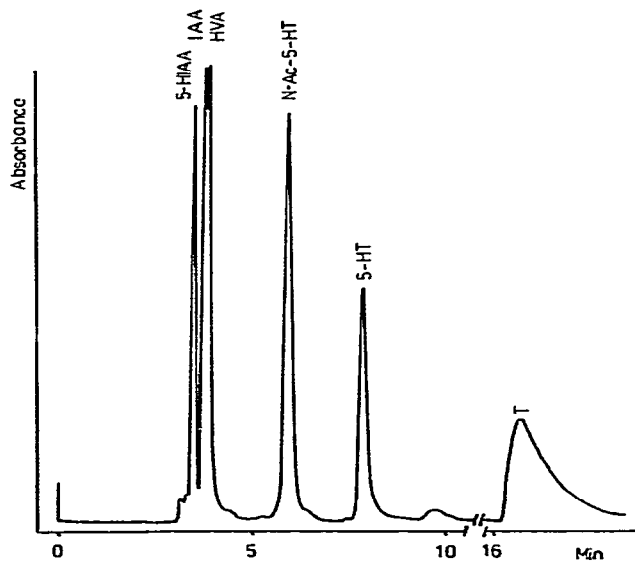

Fig. 4. Separation of six components involved in the metabolism of tryptophan with 0.1 M trichloroacetate (pH 4.70) as mobile phase.
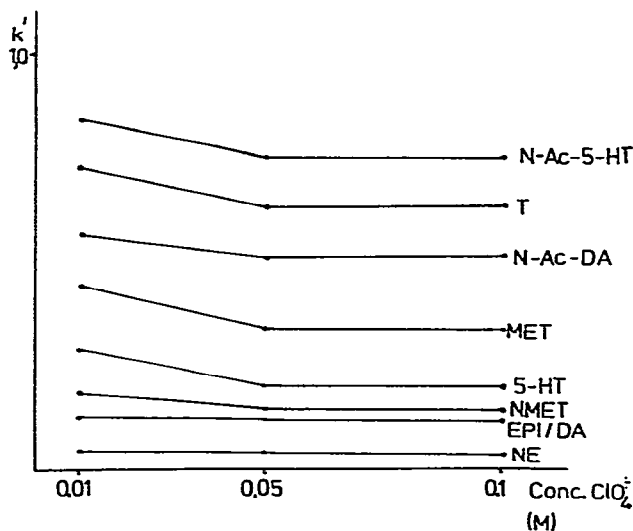
Fig. 5. Retention on μPorasil-60A at pH 2.0 as a function of ClO_4^- concentrations.

cause a high retention of basic solutes. As the charge density of silica at pH 3 is very low, large effects can be obtained by moderate changes in the cation concentration.

When 0.04–0.09 M sodium perchlorate was added to 0.01 M perchloric acid (pH 2), the retention was independent of or slightly decreased with increasing amount of perchlorate (Fig. 5). The addition of sodium perchlorate resulted in slightly sharper peaks and also in some small changes in selectivity (Fig. 6).
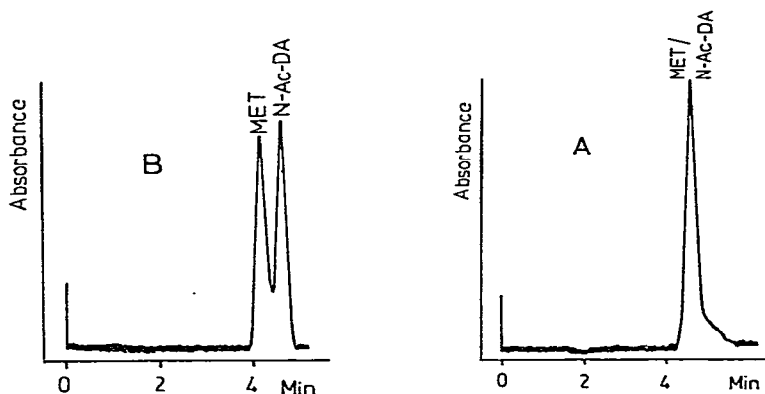
Fig. 6. Separation of metanephrine from N-acetyldopamine obtained by adding 0.04 M NaClO$_4$ (B) to 0.01 M HClO$_4$ (A) on μPorasil-60A.

Addition of organic solvents

Addition of small amounts of methanol or acetonitrile to the mobile phase strongly reduced the retention of the more hydrophobic components (Fig. 7). By adding 2% methanol to the perchlorate solvent, isomeric 7-Me-T, 5-Me-T and N-Me-T were resolved and separated from T, TRP and Me-TRP (Fig. 8). Acetonitrile
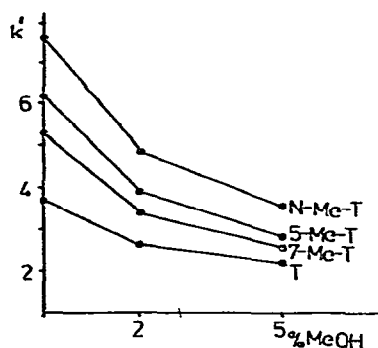
Fig. 7. Effect of addition of methanol to 0.04 *M* NaClO₄ + 0.01 *M* HClO₄ on the separation of trypt-amines on Spherisorb S5 silica.
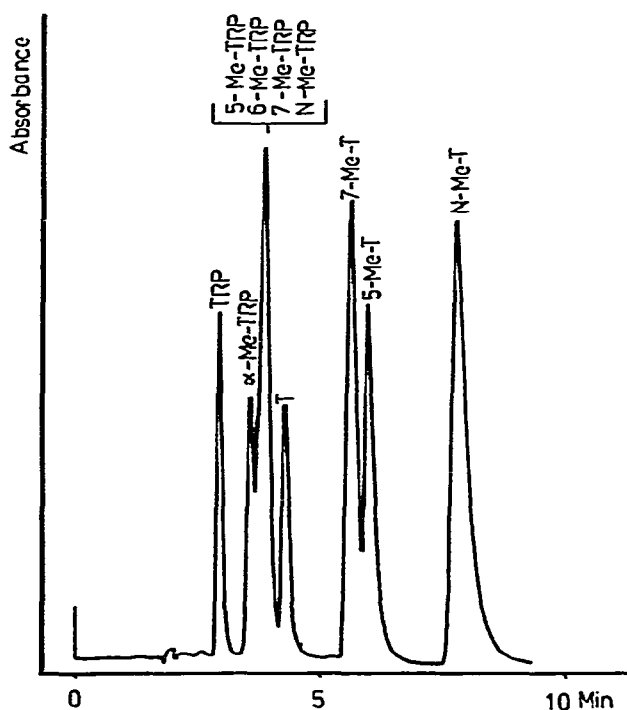

Fig. 8. Separation of tryptamines and tryptophans on Spherisorb S5-silica with 2 % methanol in 0.04 *M* NaClO₄ + 0.01 *M* HClO₄ as mobile phase.

had a greater elution strength than methanol. Addition of 2 % of acetonitrile resulted in decrease in resolution of 5-Me-T and 7-Me-T; otherwise the relative retentions were similar.

The retention of early eluting components, such as DA, NE and EPI, was increased by the addition of methanol; addition of 1 % increased the retention more than addition of 20 %.

These results are not easy to explain, but presumably indicate the existence of different retention mechanisms for the early and late eluting components.

Functional group effects

As a summary of Tables I and II and as illustrated by Figs. 9–15, the following effects were observed. Introduction of hydroxyl groups (aliphatic or aromatic) gave shorter retention. O- and N-methylation gave longer retention. N-Acetylation gave pH-dependent retention, longer at pH $\leq$ 2 and shorter at pH > 2. An increase in the alkyl chain length resulted in an increase in retention, as shown by the alcohols in Fig. 16. Carboxylic acids were eluted prior to the corresponding amides. Amino acids were eluted prior to the corresponding amines.
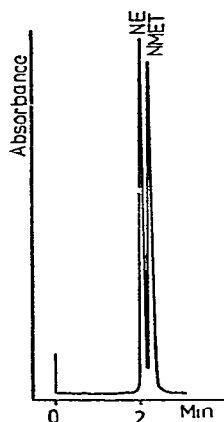

Fig. 9. Separation of the metabolite normetanephrine from its precursor norepinephrine with 0.04 M NaClO$_4$ + 0.01 M HClO$_4$ on μPorasil-60A.
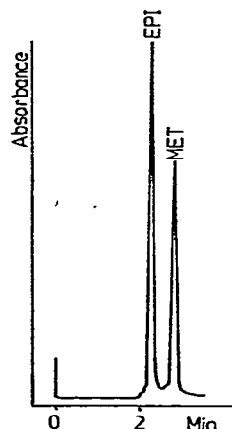
Fig. 10. Separation of the metabolite metanephrine from its precursor epinephrine with 0.04 M NaClO$_4$ + 0.01 M HClO$_4$ as mobile phase on μPorasil-60A.
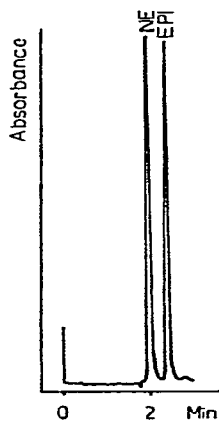

Fig. 11. Separation of norepinephrine and epinephrine with 0.04 M NaClO$_4$ + 0.01 M HClO$_4$ as mobile phase on Spherisorb S5 silica.

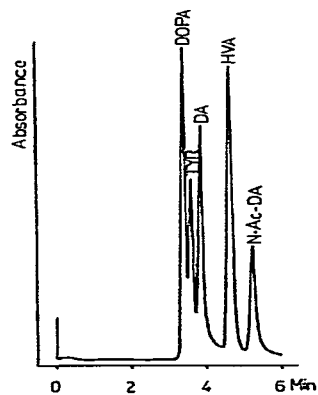Fig. 12. Separation of precursors and metabolites of dopamine with 0.04 M NaClO$_4$ + 0.01 M HClO$_4$ as mobile phase on Spherisorb S5 silica.
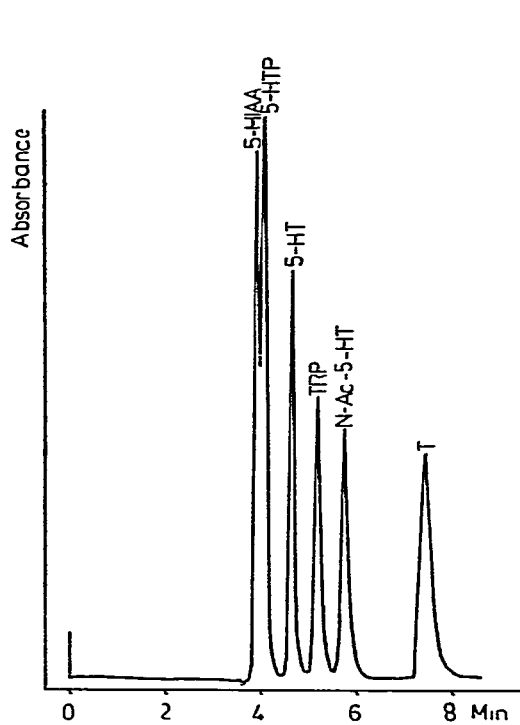
Fig. 13. Separation of metabolites of tryptophan with 0.1 M TCA as mobile phase on Spherisorb S5 silica.
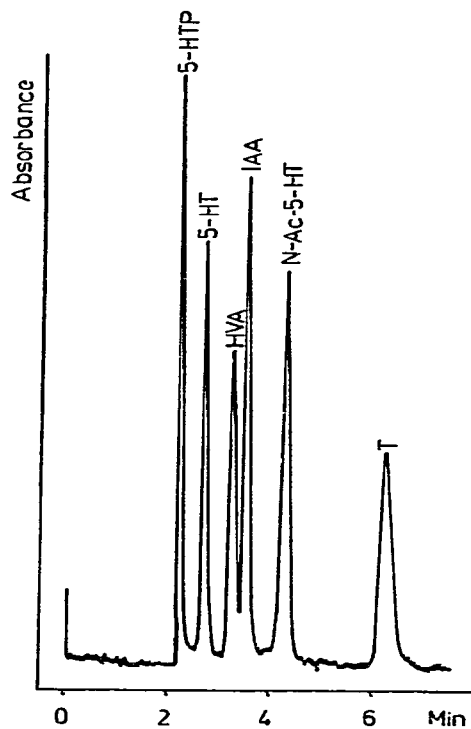
Fig. 14. Separation of indoles and metabolites with 0.04 M NaClO$_4$ + 0.01 M HClO$_4$ as mobile phase on Spherisorb S5 silica.
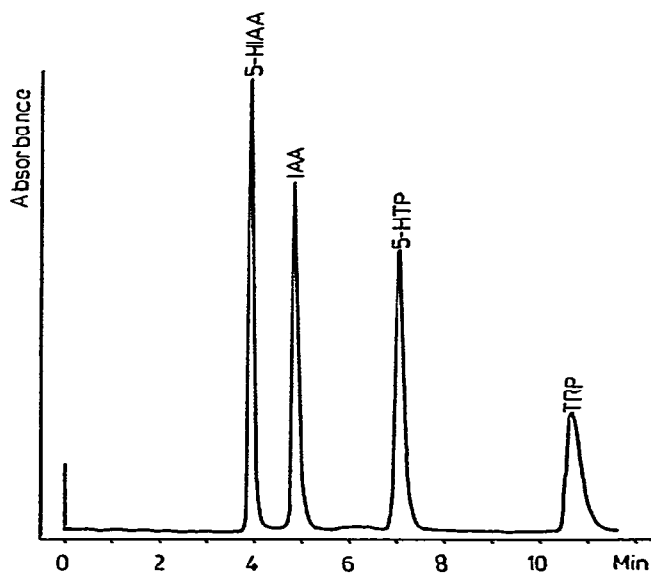
Fig. 15. Separation of four indolic acids with 0.1 M acetic acid, as mobile phase on Spherisorb S5 silica.
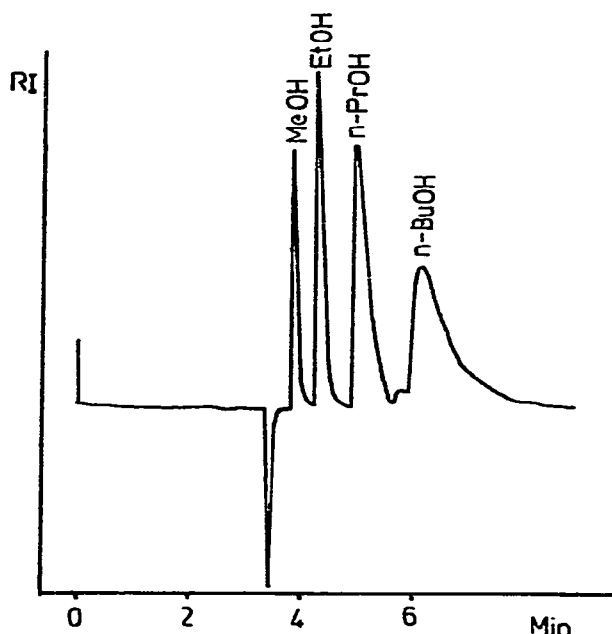
Fig. 16. Separation of alcohols with 0.04 M NaClO$_4$ + 0.01 M HClO$_4$ as mobile phase on Spherisorb silica.

Addition of dodecyl sulphate

In order to try to improve the selectivity of the early eluting catecholamines, mobile phases consisting of 0.01 M dodecyl hydrogen sulphate in 0.1 M formic acid at pH 2.5, 3.0 and 3.4 were tested on NE, DA and EPI. The retention was slightly lower than in 0.1 M formic acid alone, the peak shape at higher pH was only slightly improved and no separation of DA and EPI was obtained.

As the addition of an ion-pair reagent with a C_{12} chain did not increase the retention, the conclusion that can be drawn is either that NE, DA and EPI did not form ion pairs or that the formation of ion pairs had little effect on the retention.

A similar retention of the three components was actually obtained by Crommen[8] with camphor-10-sulphonate as the assumed ion-pairing reagent and yet the conclusion was drawn that ion-pair formation could be used to regulate (by increasing) the retention of the most hydrophilic biogenic amines on aqueous silica. Based on the experimental results this conclusion cannot be accepted.

Retention mechanisms

As can be seen from the effects of different functional groups, a reversed-phase elution order was generally observed, in agreement with the findings of Crommen[8]. A decrease in the retention of hydrophobic compounds as a result of adding organic solvents also supported the concept of a quasi-reversed-phase system. The pH effects were generally in line with, but could not be explained solely by, "reversed-phase partition".

As "reversed-phase partition" requires a stationary phase that is less polar

than the mobile phase, the postulated model contains a stationary phase of water adsorbed on silica and a mobile phase of the aqueous acid with or without an organic solvent. At low pH (1–2) the retention of moderately and highly retained components depends mainly on the partition coefficients between the two liquid phases, without direct contact with the silica surface. At higher pH coulombic forces can compete with and also overshadow the effect of Van der Waals forces, owing to direct contact with silica caused by a reduction in the amount of adsorbed water. The behaviour of components with low retentions cannot be satisfactorily explained by this model, which probably means that hydrophilic compounds can compete directly with water for the silica surface functions.

The concept of partition chromatography without direct interactions with the silica surface is supported by the fundamental studies of Scott and co-workers[11–13] on solute–solvent interactions on silica. The different mechanisms for hydrophilic components is also in full agreement with this work.

The appearance of ion-exchange effects on increasing the pH is supported by the fact that increased ionization of silanol groups reduces the amount of hydrogen-bonded adsorbed water[14]. The experimental evidence was found in the strong retention of basic solutes and the low retention of acidic solutes. As a natural consequence of the reduced volume of the stationary phase, the retention of neutral solutes decreased. The postulated model does not pretend to behave like a genuine reversed-phase system. Thus, the "salting-out effect" on adding salts to the mobile phase is not expected, and was not found, at least with the moderate concentrations examined.

Crommen[8] showed that the retention of a number of different amines on aqueous silica was increased by adding ion-pair reagents. The results of this study show that the same is generally not true for catecholamines and indoleamines. Except for a predominantly reversed-phase elution order, the retentions of amines, amino acids and acids on aqueous silica cannot be described by one general mechanism.

The present conclusion is that quasi-reversed-phase partition, adsorption/ion exchange and ion-pair formation may contribute to the retention, depending on the nature of the solute, the pH and the content of acids/ions in the mobile phase.

Silica

The sample retention is usually[8], but not necessarily, related to the specific surface area of the silica. Thus, the 10-μm μPorasil-60A packing with a surface area of 500 m^2/g generally gave lower retentions than the 5-μm Spherisorb S5 packing with a surface area of 220 m^2/g. The μPorasil-60A packing had been deactivated by the manufacturer, a procedure that is supposed to have reduced the density of silanol groups. Since water is adsorbed to the silanol groups and not to the hydrophobic siloxane surface[12], the reduction in the amount of adsorbed water should result in decreased retention. Owing to the higher retention, better resolution was normally obtained on the Spherisorb S5 column. With silica of higher surface area (600–800 m^2/g) improved resolution of the hydrophilic catecholamines is expected. A study of this and of the combination of silica with a cation exchanger is in progress.

Reproducibility and column lifetime

Based in 10–20 measurements on each component, the capacity factors in Tables I and II were calculated with a relative precision (percentage standard devia-

tion of the mean) of better than 2 % over a 10-h measuring period and better than 5 % over a 2-week measuring period at pH 2. At pH > 2 the short-term precision was lower (better than 10 %).

Over an 18-month trial period with the same columns the retention of basic components decreased slowly, the greatest decrease being for the basic highly retained compounds, such as a 25 % reduction per year for tryptamine. This is the reason why N-Ac-5-HT changed place with T in Fig. 14 compared with Fig. 1. After 1 year the void volume of the Spherisorb S5 column had increased by 5 %.

The column efficiencies were maintained at the same high level over the 18-month trial period.

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