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REVERSED-PHASE SYSTEMS FOR THE ANALYSIS OF CATECHOLAMINES AND RELATED COMPOUNDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Phase systems using alkyl-modified silica as an adsorbent, used as such and as a support for dynamically coated ion exchangers, were investigated for their capability in separating catecholamines and related compounds.

Simple reversed-phase adsorption chromatography with C_8-bonded silica is not able to separate these compounds very well because of (i) the very small retention of the more basic compounds in circumstances where the acidic compounds are well separated, (ii) bad peak shapes and (iii) low column efficiencies, although the last drawback can be circumvented by the addition of inorganic anions to the eluent. The addition of a dynamically coated cation exchanger, sodium dodecylsulphate (SDS), to the eluent not only brings about drastic changes in the selectivity, but also makes available an additional degree of freedom for influencing the selectivity. The retention of the basic solutes increases upon addition of SDS and the retention becomes inversely proportional to the counter ion (Na^+) concentration. Further, it was found that columns previously loaded with SDS can be used with SDS-free eluents when a pre-column, loaded with SDS, is used or with eluents containing a very small amount of SDS (<0.001 %, w/v). These SDS-coated phase systems behave similarly to phase systems containing SDS in the eluent and show a better column stability and UV background.

INTRODUCTION

The determination of the concentrations of catecholamines and related compounds in body fluids has become extremely important for the recognition and understanding of metabolic disorders that are associated with pathological conditions such as hypertension[1], certain psychic syndromes[2] and adrenal and neuronal tumours[3,4] (pheochromocytoma or neuroblastoma). Many techniques have been examined for use in the analysis of catecholamines and related compounds in body fluids. Fluorimetry[5,6], thin-layer chromatography (TLC)[7] and gas chromatography (GC)[8,9] have been applied. The fluorimetric methods are non-selective and only permit the measurement of a group of catecholamines together[10]. Only in some instances can a certain

degree of selectivity be obtained by selective oxidation procedures[5]. TLC is suitable for qualitative analysis but is restricted in its quantitative reliability. When applying GC volatile derivatives have to be formed, which introduces a high degree of uncertainty into the quantitative analysis.

High-performance liquid chromatography does not suffer from the above disadvantages and has been found to be suitable for the analysis of the catecholamines and related compounds, in particular when combined with selective measurement methods with a low detection limit, such as fluorimetric[11] or electrochemical detection[12–15].

Ion-exchange[16], adsorption[11,17,18] and ion-pair chromatography[19,20] have been applied successfully to this problem. All of these liquid chromatographic (LC) methods described so far involve the separation and analysis of just a few catecholamines or related compounds in body fluids. However, a large number of metabolites are formed in the body and the analysis of all of these compounds might provide valuable information about the metabolism in relation to certain pathological conditions. Therefore, we thought it useful to devise a chromatographic phase system that is capable of separating simultaneously a large number of these compounds, both acidic and basic.

Of all the phase systems described in literature, the reversed-phase systems showed the best selectivity and column performance. Especially when applying dynamic ion exchange[15,17,18,21,22], excellent selectivity can be obtained. The results of the studies on this topic are presented in this paper.

EXPERIMENTAL

Apparatus

All chromatographic experiments were carried out on a high-pressure liquid chromatograph (1010A LC, Hewlett-Packard) using UV detection (1030B, Hewlett-Packard) or a home-made coulometric detector[23], a high-pressure sampling valve (Valco CV-6-UHpa) and a thermostated (25°) stainless-steel column of dimensions 250 × 2.8 mm I.D. fed by a thermostated water-bath (Haake NB 22). In some experiments a pre-column of dimensions 100 × 10 mm I.D. was installed in front of the injection valve. The wavelength of the UV detector was adjusted to 280 nm.

Materials

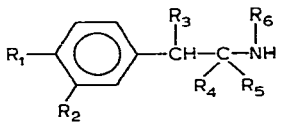
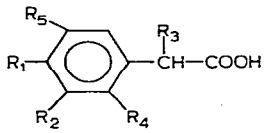
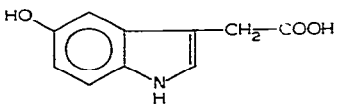
All solvents were of analytical-reagent grade and were used without further pre-treatment. The column materials used were commercially available octyl-modified silica (RP-8, mean particle size 5 or 10 μm; Merck, Darmstadt, G.F.R.). Sodium dodecylsulphate (SDS) was obtained from Merck. The catecholamines and related compounds were commercial products (Sigma, St. Louis, Mo., U.S.A., and Aldrich, Milwaukee, Wisc., U.S.A.). Their structures and the abbreviations used henceforth are given in Table I.

Procedures

The separation columns were packed by a pressurized slurry method[24] using a mixture of chloroform and tetrabromoethane of specific gravity 1.82 or tetrachloroethane as the slurry liquid. The columns were washed successively with 100 ml of

TABLE I

STRUCTURES AND ABBREVIATIONS OF CATECHOLAMINES AND RELATED COMPOUNDS

Compound	*Structure*	*Abbreviation*	R_1	R_2	R_3	R_4	R_5	R_6
3-Methoxytyramine		3-M-Tyrm	OH	OCH_3	H	H	H	H
Tyramine		Tyrm	OH	H	H	H	H	H
Dopamine		3-H-Tyrm	OH	OH	H	H	H	H
Isoprenaline		Isopren	OH	OH	OH	H	H	C_3H_7
Metanephrine		MN	OH	OCH_3	OH	H	H	CH_3
Normetanephrine		NM	OH	OCH_3	OH	H	H	H
Synephrine		Syn	OH	H	OH	H	H	CH_3
Norsynephrine		NSyn	OH	H	OH	H	H	H
3,4-Dihydroxyphenylalanine		DOPA	OH	OH	H	H	COOH	H
Epinephrine		E	OH	OH	OH	H	H	CH_3
Norepinephrine		NE	OH	OH	OH	H	H	H
Homovanilmandelic acid		HVA	OH	OCH_3	H	H	H	--
3,4-Dihydroxyphenylacetic acid		DOPAC	OH	OH	H	H	H	--
2,5-Dihydroxyphenylacetic acid		HGA	H	H	H	OH	OH	--
Vanilmandelic acid		VMA	H	OCH_3	OH	H	H	--
3,4-Dihydroxymandelic acid		DHMA	OH	OH	OH	H	H	--
5-Hydroxyindole-3-acetic acid		5-HIAA						

methanol, 100 ml of propanol and finally with the eluent until constant retention was achieved. The pre-column was packed by a dry-packing technique.

The preparation of the SDS loaded columns was as follows. Freshly packed pre- and analytical columns (washed with methanol and propanol) of Rp-8 were connected in series and pumped through with a citrate-buffered water–propanol mixture (97:3, v/v) (pH 1.6), containing a certain amount of SDS (usually 0.3%, w/v). Then the pre-column and analytical column were separately pumped through with 1000 ml of eluent of the same composition but without SDS. The pre- and analytical columns were subsequently installed in the liquid chromatograph and were ready for use.

RESULTS AND DISCUSSION

In order to determine optimal chromatographic conditions for the separation of the relevant compounds, the retention behaviour of these substances, including 5-HIAA because of its importance in psychiatry in conjunction with some catecholamine derivatives, was studied in two reversed-phase modes; (i) octyl-modified silica as adsorbent in combination with buffered water–propanol mixtures; and (ii) solvent-

generated cation-exchange systems using sodium dodecylsulphate (SDS) as anionic surfactant.

In the *normal reversed-phase* operation the retention can be adjusted, within certain limits, by variation of the propanol concentration (see Fig. 1) and for the acidic solutes also by the pH of the eluent. The retention of the basic solutes increases sharply at pH > 6–7 but unstable columns were obtained in alkaline medium. Moreover, the catecholamines (basic) are easily oxidized at high pH[25]. In order to retain the basic solutes significantly, very low organic modifier concentrations have to be used (<0.3%). The selectivity of these phase systems, however, is poor for the basic compounds investigated. Moreover, the peak shape for most substances in these normal reversed-phase systems was asymmetric, probably as a result of a strong interaction of the very polar groups in these molecules with non-silanized sites of the octyl-modified support. The addition of sodium perchlorate (0.05–0.2 *M*) diminished the peak tailing significantly and improved the column efficiency (also of the acidic solutes). The favourable effect of sodium salts of inorganic acids on peak tailing was observed earlier in our laboratory when separating organo-mercury complexes[26]. Moreover, the addition of sodium perchlorate influences differently the retention of acidic and basic compounds, as can be seen from Fig. 1. The increase in the retention of the basic compounds when sodium perchlorate is added to the eluent is not well understood. Probably perchlorate–solute ion pairs are formed in the mobile phase, which favours the distribution into the interface.

Despite the favourable effect of sodium perchlorate, no complete rapid and

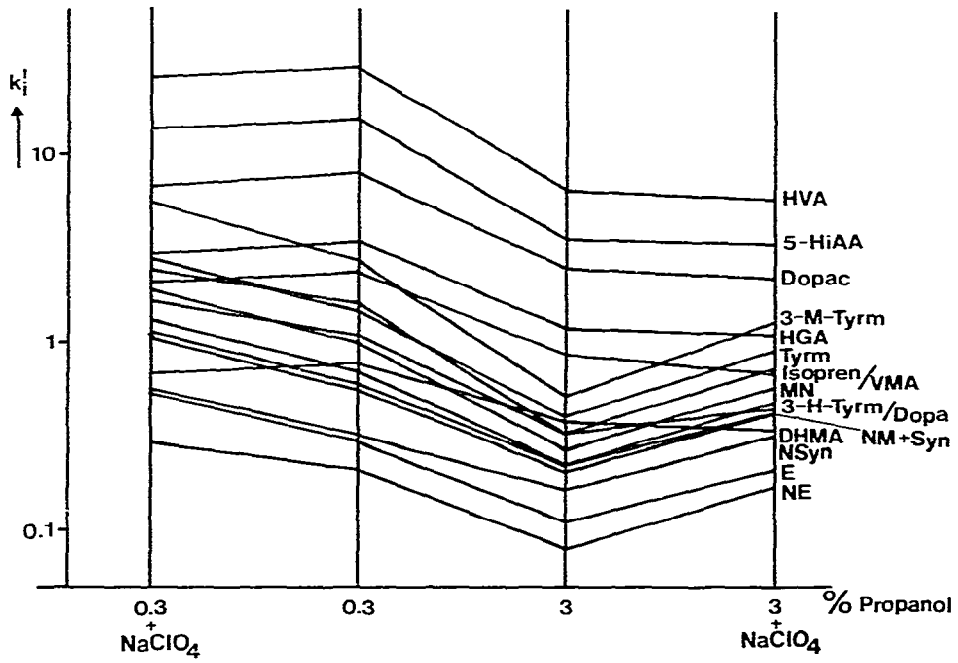


Fig. 1. Influence of propanol concentration and sodium perchlorate addition on the capacity ratio (k'_i) of catecholamines and related compounds. Stationary phase: C_8-bonded silica. Mobile phase: 0.02 *M* citrate (pH = 1.65) + *n*-propanol (0.3–3%, v/v) + $NaClO_4$ (0–0.2 *M*), $T = 25°$.

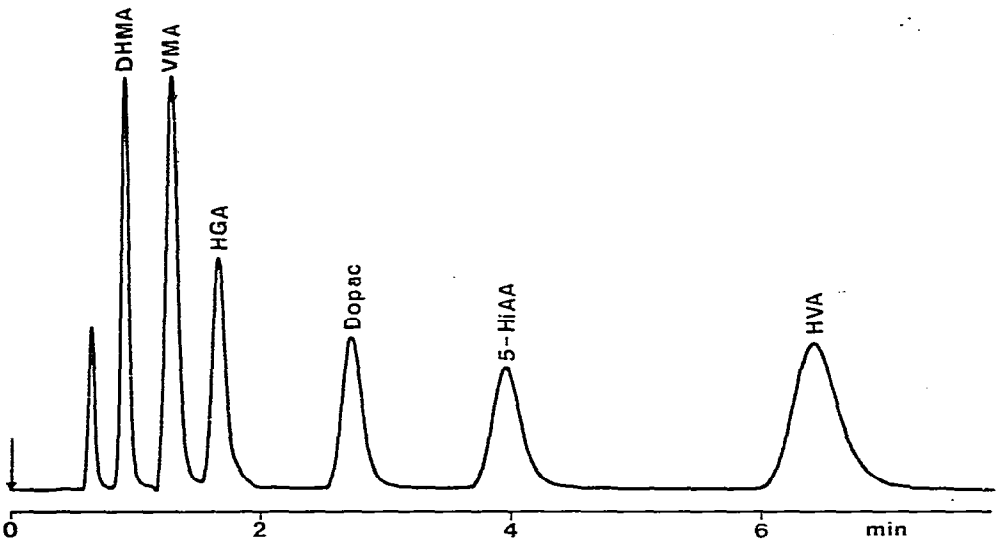


Fig. 2. Rapid separation of a test mixture of acidic catecholamine metabolites on a normal reversed-phase system. Stationary phase: C_8-bonded silica. Column length: 250 mm. Mobile phase: 0.02 *M* citrate (pH = 2.5) + 3% (v/v) propanol + 0.2 *M* $NaClO_4$, $T = 25°$, $\Delta P = 120$ bar.

isocratic separation of all catecholamines and related compounds can be achieved on these normal reversed-phase systems. However, by adaptation of the propanol content, pH and perchlorate concentration, a complete separation of the acidic solutes as well as some of the basic compounds can be achieved, as shown in Figs. 2 and 3.

In order to investigate the type of organic modifier, methanol and acetonitrile were also tested. No significant selectivity changes were found between the three organic modifiers. With respect to the column stability, however, propanol was far superior.

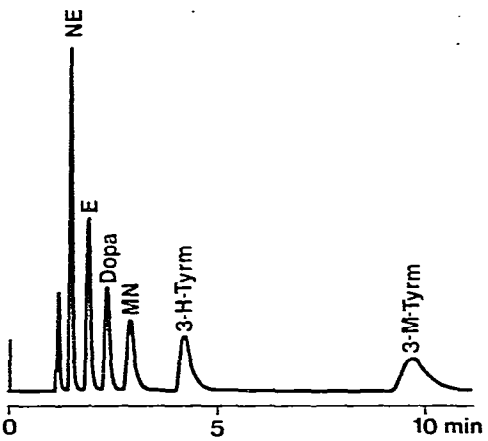


Fig. 3. Separation of a test mixture of the basic catecholamines and some metabolites on a normal reversed-phase system. Stationary phase: C_8-bonded silica. Column length: 250 mm. Mobile phase: 0.02 *M* citrate (pH = 2.5) + 0.3% (v/v) propanol + 0.2 *M* $NaClO_4$, $T = 25°$, $\Delta P = 120$ bar.

In conclusion, normal reversed-phase systems using octyl-modified silica in combination with buffered propanol mixtures containing sodium perchlorate up to 0.2 *M* is suitable for the separation of acidic catecholamine metabolites but are less suitable for the separation of all basic compounds.

Dynamic cation-exchange systems using anionic surfactants were found to be suitable for the separation of amines such as amino acids and catecholamines[15,21,22]. The effect of addition of SDS to the eluent on the retention of the acidic and basic compounds is shown in Fig. 4. As can be seen, the addition of SDS (0.03%) greatly enhances the retention of the basic solutes and reduces the retention of acidic metabolites slightly. Due to the presence of SDS in the eluent, a layer of SDS is formed at the hydrophobic column support, which has the ability to exchange its counter ion (*e.g.*, Na^+) with solute cations[22,27]. Apart from retention via a cation-exchange mechanism, also a normal physical distribution between the mobile phase and the interface can occur[27]. For basic solutes retention via a cation-exchange mechanism predominates, while for acidic solutes physical distribution is the main retention mechanism.

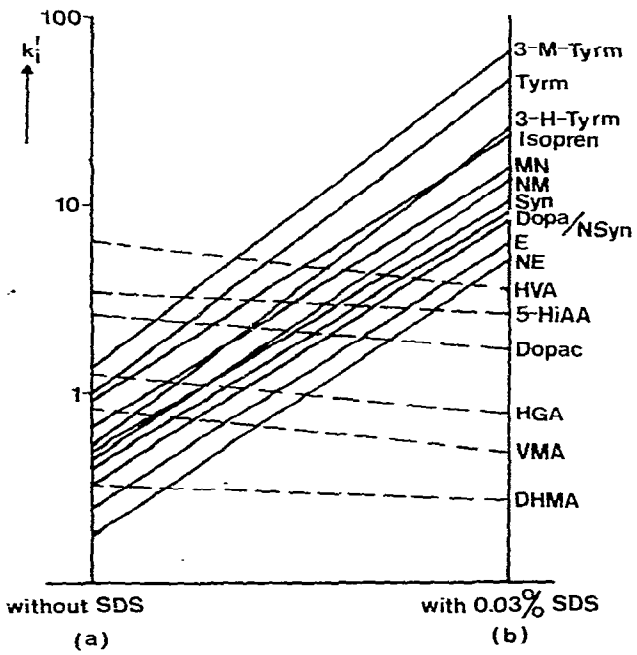


Fig. 4. Effect of sodium dodecylsulphate (SDS) on the capacity ratio of catecholamines and related compounds. Stationary phase: C_8-bonded silica. Mobile phase: (a) as in Fig. 2; (b) 0.02 *M* citrate (pH = 2.5) + 3% (v/v) propanol + 0.125 *M* $NaClO_4$ + SDS (0.03% w/v). $T = 25°$.

The presence of SDS in the phase system introduces a number of extra parameters for adjusting the degree and order of retention, such as the SDS and counter ion concentration[18,21,22,27]. Dynamic ion-exchange systems behave like conventional ion-exchange systems, so that the retention can be influenced, in a predictable way, by adjustment of the counter ion concentration, as is shown for NE and E in Fig.

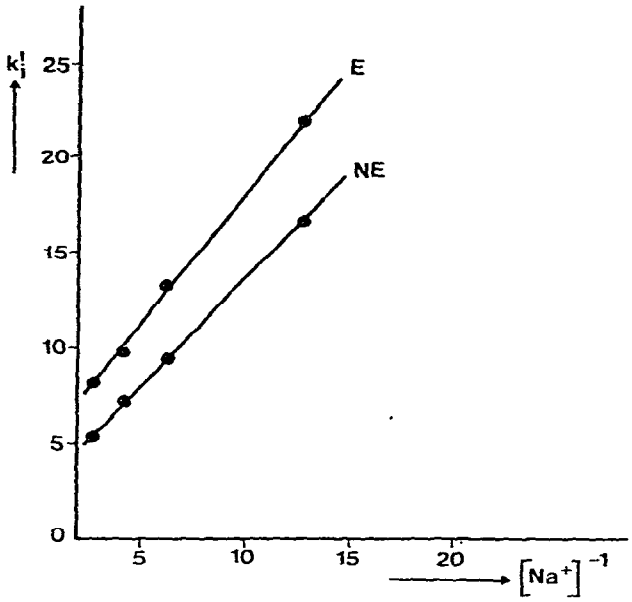


Fig. 5. Dependence of the capacity ratio of NE and E on the counter ion (Na^+) concentration in the mobile phase. Stationary phase: C_8-bonded silica. Mobile phase: 0.02 *M* citrate (pH = 2.5) + 1% (v/v) propanol + $NaClO_4$ (0.08–0.4 *M*) + 0.3% (w/v) SDS, $T = 25°$

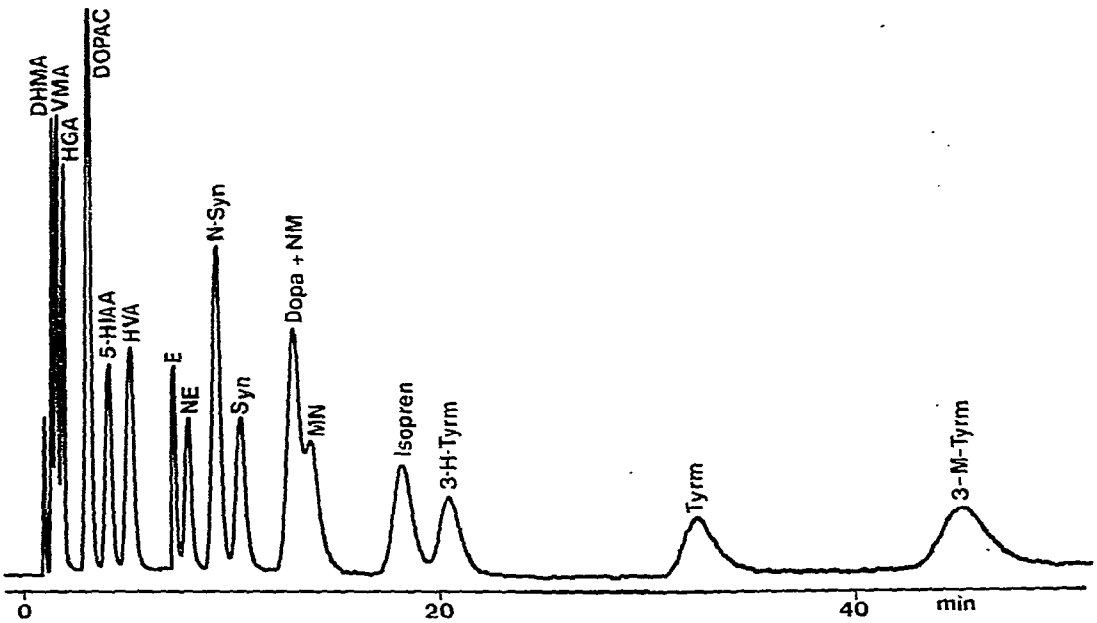


Fig. 6. Separation of a test mixture of acidic and basic catecholamines and related compounds under isocratic conditions on a dynamic cation-exchange system. Stationary phase: C_8-bonded silica. Column length: 250 mm. Mobile phase: as in Fig. 5, $\Delta P = 120$ bar.

5. The counter ion concentration is a powerful parameter for adjusting the retention of the basic solutes in these dynamic cation-exchange systems. With respect to the effect of the type of counter ion, it can be noted that K^+ cannot be used as it forms insoluble salts with SDS. However, cations such as Mg^{2+}, $(CH_3)_4N^+$, $C_2H_5NH_3^+$ and $C_6H_{11}NH_3^+$ can also be applied and might introduce slight selectivity changes. In particular, the last cation acts as a very strong counter ion and is worth investigating more extensively.

By adaptation of the different parameters available, a nearly complete separation of the acidic and basic compounds can be achieved in about 40 min, as is shown in Fig. 6. As higher ultimate pressures can be used, it can be expected that the separation time could be reduced to less than 20 min.

As can be seen from Fig. 6, under the chosen conditions the acidic solutes are eluted before the basic compounds, which is the reverse order to that obtained in normal reversed-phase systems. The resolution of the acidic compounds, however,

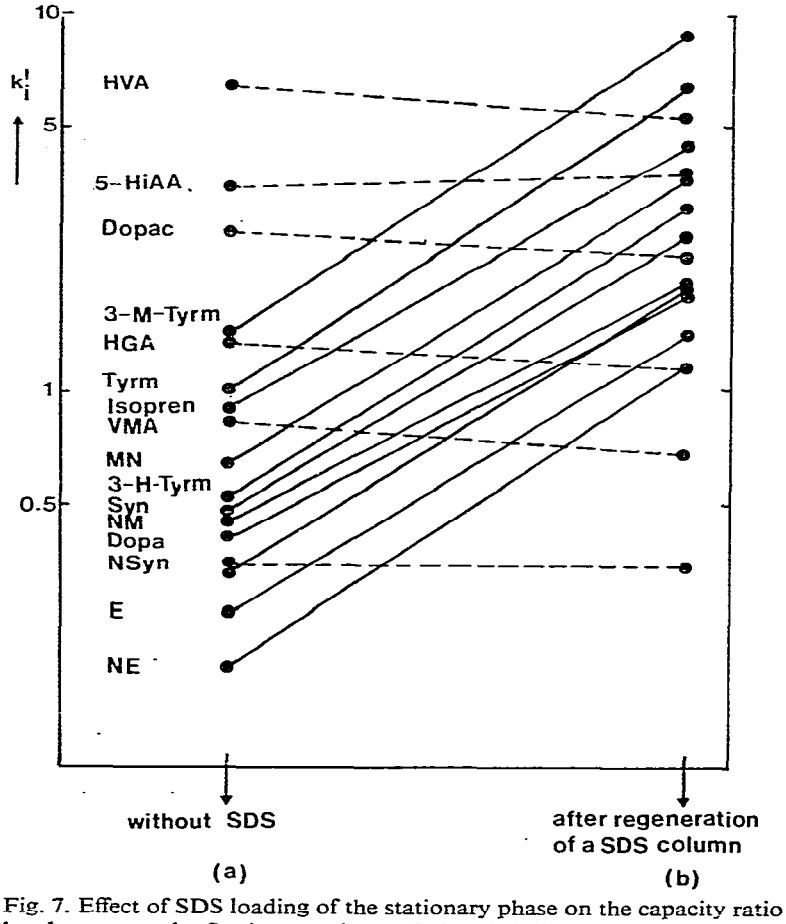


Fig. 7. Effect of SDS loading of the stationary phase on the capacity ratio of catecholamines and related compounds. Stationary phase: C_8-bonded silica, loaded with SDS as described in the text. Mobile phase: (a) as in Fig. 2; (b) 0.02 *M* citrate (pH = 2.5) + 3% (v/v) propanol + 0.2 *M* $NaClO_4$, $T = 25°$.

is worse on the dynamic cation-exchange systems than that obtained in normal reversed-phase systems (*cf.*, Fig. 2). The separation of these acidic compounds can be improved by decreasing the propanol or SDS concentration in the eluent. However, a decrease in the propanol concentration results in an increase in the retention of the basic solutes[22], while a decrease in the SDS concentration results in a decrease in the retention but also in the resolution of these basic compounds. Therefore, a compromise has to be found, depending on the separation problem (*i.e.*, which and how many metabolites have to be separated.)

The presence of SDS in the eluent is favourable for the basic solutes and less for the acidic solutes. A phase system in which the stationary phase is loaded with SDS but its concentration in the mobile phase is low might be advantageous. In order to create such conditions, a column filled with RP-8 was loaded with SDS by elution with propanol–water (3:97) containing 0.3% (w/v) of SDS. Then the eluent was changed to the same mixture but without SDS and the retention of the solutes were measured after a certain equilibration time. The results of these measurements, including the capacity factors on the normal reversed-phase system, are shown in Fig. 7. As can be seen, a large increase in the retention of the basic compounds occurs compared with that on the normal-phase system, while the capacity ratios of the acidic solutes do not change much. This result indicates that SDS is still present

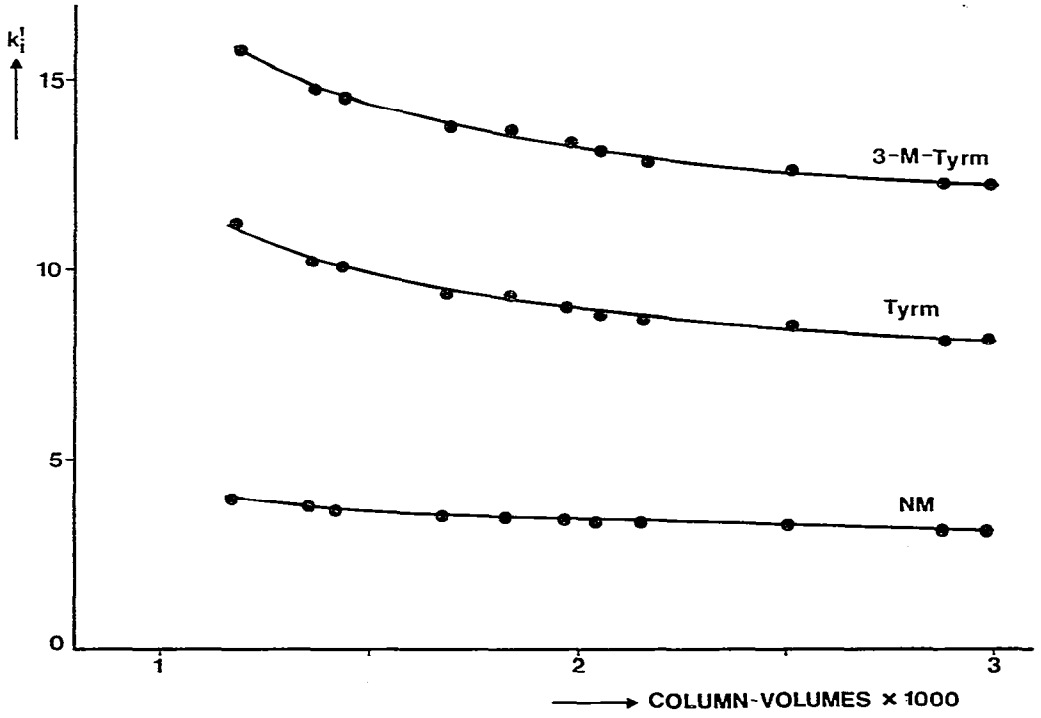


Fig. 8. Course of the capacity ratio of 3-M-Tyrm, Tyrm and NM as a function of the eluted number column volumes of eluent on an SDS-coated column. Stationary phase: C_8-bonded silica loaded with SDS as in Fig. 7. Mobile phase: 0.02 *M* citrate (pH = 1.65) + 3% (v/v) propanol + 0.2 *M* $NaClO_4$, $T = 25°$.

in the interface and that cation exchange occurs for the basic compounds even when no SDS is present in the eluent. This result agrees with those previously obtained with solvent-generated anion-exchange systems[27] and suggests a retention mechanism based on cation exchange rather than on adsorption of SDS–solute ion pairs, formed in the mobile phase[18].

Comparison of Figs. 4 and 7 shows that the capacity ratios of the basic solutes are smaller in the phase system without SDS in the eluent compared with the phase system with SDS present in the eluent. The smaller capacity ratios when no SDS is present in the eluent is caused by stripping off of SDS from the column support during the equilibration time, as can be seen from Fig. 8. However, a more or less steady-state situation occurs after elution of a large number of column volumes of eluent. From Fig. 8, it must be concluded that even under steady-state conditions a certain SDS concentration exists in the mobile phase (*i.e.*, equilibrium concentration), which is of the order of 0.001–0.005% (w/v). No change in capacity ratios occurred when a pre-column, loaded and equilibrated similarly to the analytical column, was installed in front of the injection valve or when eluents were used that were pumped through a steady-state analytical column.

Repeated loading of the same column and applying the same equilibration procedure gave variations in the capacity ratios of less than 5%. Fig. 9 shows a chromatogram of the separation of the acidic and basic solutes on these SDS-loaded columns. Although no complete separation is achieved, the phase system looks promising because of the resolution of the acidic solutes and the possibility of shifting the basic solutes by variation of the type and concentration of the counter ion. An

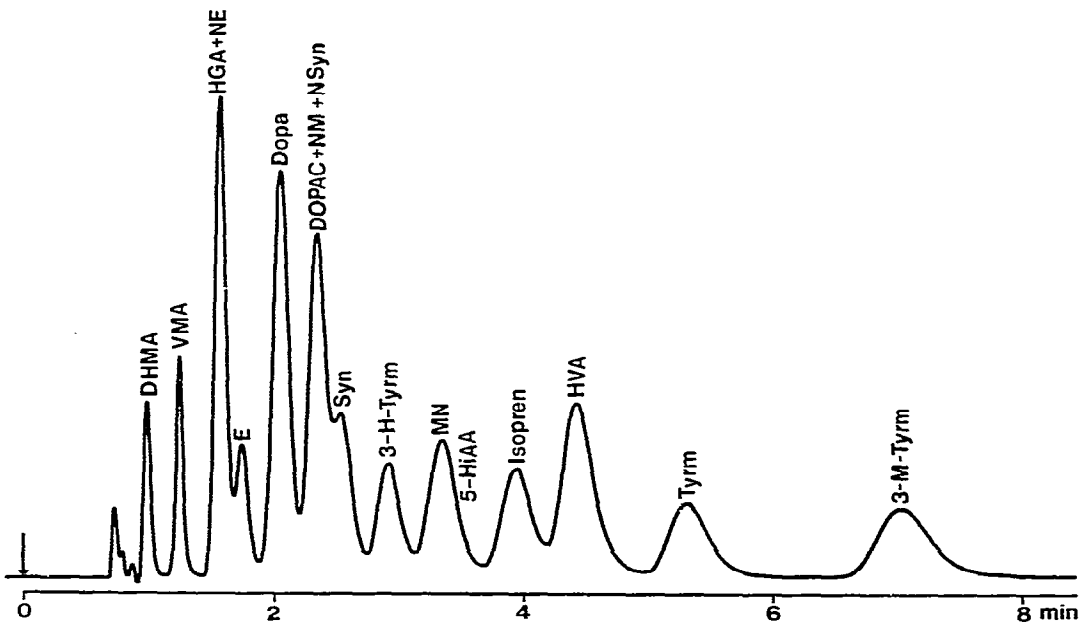


Fig. 9. Separation of a test mixture of catecholamines and related compounds on an SDS-coated column. Conditions: as in Fig. 7; column length, 250 mm, ΔP = 120 bar.

additional advantage might be the smaller background absorbance, caused by SDS, when using UV detection.

Both dynamic cation-exchange systems show excellent peak symmetry for all solutes investigated and no difference in column performance was found. However, the SDS free eluent phase systems were found to be more stable with respect to lifetime. Experiments with electrochemical detection[23] were also carried out. A typical chromatogram obtained with this detection method is shown in Fig. 10. However, difficulties were encountered caused by significant amounts of metal ions in the eluent, apparently as the result of corrosion of different parts of the stainless-steel apparatus by the eluent, especially when citrate buffers were used. The practical realization of the LC–electrochemical detection combination for these compounds should be investigated further.

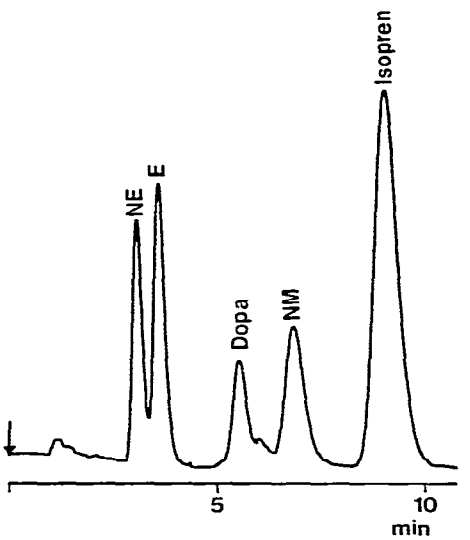


Fig. 10. Separation of catecholamines and some related compounds (100 ng of each) using electrochemical detection. Stationary phase: C_8-bonded silica loaded with SDS as in Fig. 7. Mobile phase: 0.02 *M* citrate (pH = 2.0) + 3% (v/v) propanol + 0.02 *M* $NaClO_4$ + 0.001% (w/v) SDS. $T = 25°$. Electrochemical detection: 0.75 V *vs.* Ag–AgCl electrode.

This work has shown that dynamic cation-exchange systems constitute a selective and versatile phase system for the separation of catecholamines and related compounds. The predictable relationship between the chemical structure of the solute and changes in the eluent composition (*e.g.*, counter ion concentration, addition of SDS) constitute a definite advantage.

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