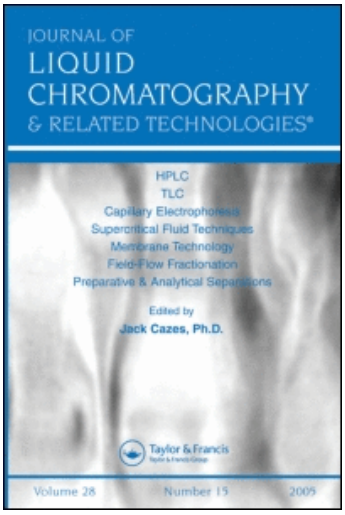

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THE INFLUENCE OF MOBILE PHASE ACIDITY AND COUNTERION TYPE AND CONCENTRATION ON THE RETENTION BEHAVIOUR OF URINARY CATECHOLAMINES AND SAMPLE IMPURITIES IN ION-PAIR REVERSED-PHASE LIQUID CHROMATOGRAPHY

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ABSTRACT

Octyl silica reversed-phase material was used as a support for dynamically coated anion surfactant, octyl sulphate, acting as an ion-pairing agent for retention of biogenic amines. In this system the influence of counterion type and concentration on retention behaviour of the analytes was studied. The strongest competitive effect of all counterions studied /Li, Na, K, NH_4/ exerted potassium and ammonium. However, the effect of potassium was lower than expected according to known value of relative selectivity. The accompanying anion and pH in range of 3,6 to 5,5 did not influence the retention noticeable. Regarding determination of catecholamines in urine after a rapid alumina clean-up, a simple ammonium acetate mobile phase of pH 5,4 gave best results with respect to separation of amines from interfering substances present in the sample.

INTRODUCTION

The most widely used packing materials for high - performance liquid chromatography /HPLC/ today are the silica gels, modified with non-polar hydrocarbons, the reversed-phase /RP/ materials. The retention time of non-polar molecules is directly proportional to the chain length of chemically bonded hydrocarbon, usually with 8 or 18 carbon atoms in length /C_8 or C_{18} columns/, and the degree of coverage achieved. With regard to determination of catecholamines the RP chromatography with C_8 or C_{18} bonded silica is not able to separate these compounds very well because of the very small retention. However, the column sorbent can be used as a support for dynamically coated ion exchangers, such as the ion-pairing /IP/ anion surfactant sodium dodecyl sulphate /SDS/ or octyl sulphate /OS/. Due to the presence of IP agent in the mobile phase, a layer of it is formed at the hydrophobic column support, which has the ability to exchange its counterion, e.g. sodium, with solute cations. The retention of the basic solutes increases upon addition of IP agent and the retention becomes inversely proportional to the counterion concentration. Thus, the dynamically coated cation exchangers make available an additional degree of freedom for influencing the selectivity /1/.

In this study we were interested in the findings of how to influence the kind and concentration of a counterion and pH of the mobile phase, the retention behaviour of catecholamines and some of the interfering substances after a simple alumina urine cleanup. The competitive effect of a counterion should be in a close relationship with its known selectivity toward the particular ion exchange group. The use of sodium as mobile phase cation prevails as, e.g., urinary catecholamines could be determined in such a system after a simple alumina cleanup /2/.

Some authors recommend potassium in connection with quantitation of plasma catecholamines because of its highest tabelated resin affinity among the monovalent cations tested /3/. Also ammonium cation proved to be very useful as simple ammonium acetate systems can often replace complicated and expensive buffers /4/. Next to this, we examined also lithium as a cation with the lowest relative selectivity to the exchanger. The studies were performed with RP octyl silica /10 µm/ column.

MATERIALS AND METHODS

Inorganic salts of lithium, natrium, potassium and ammonium acetate, phosphate or citrate, acetic and citric acid, all in the best analytical grade, ethylenediaminetetraacetic acid disodium salt /EDTA/ and acetonitrile were obtained from Lachema /Brno, Czechoslovakia/. Alumina /acidic grade I, 70-230 mesh/ and TRIS were delivered by E. Merck /Darmstadt, FRG/. The catecholamines, norepinephrine, epinephrine and dopamine /NE, E, DA/ and 1-octanesulfonate /OS/ were obtained from Sigma /St. Louis, MO/. The stock solution of catecholamines was prepared in 0,5 mol/l acetic acid with 0,1% EDTA and sodium metabisulfite as antioxidant.

Separations were performed on a C_8 RP column /250 x 4,6 mm, 10 µm silica particle size/ from Serva /Heidelberg, FRG/. The HPLC system consisted of Rheodyne 7125 injection valve with 200 µm sample loop, solvent delivery pump FR-30 /Knauer, FRG/ with pulse dampener and electrochemical detector ED 641 VA /Metrohm, Switzerland/. The detector was operated at + 0,7 V with an Ag/AgCl reference electrode and glassy carbon working electrode at 5 nA full scale range. Mobile phase consisted of various salts /see Table 1 and Figures 1 and 2/ in aqueous solution with 2% acetonitrile, 100 mg/l EDTA and 100 mg/l OS

TABLE 1

Retention of Biogenic Amines in Dynamic Cation-Exchange Systems in Relation to the Type of Cation and its Concentration in the Mobile Phase. Column: 250 x 4,6 mm RP-8, Mobile Phase: Aqueous Buffer Solutions with 0,1 g OS, 0,1 g EDTA and 2% Acetonitrile. Flow-rate: 0,8 ml/min.

Type of Cation	Relative selectivity[a]	Cation concentration (mmol/l)	Kind of accompanying anion	pH	Retention time (min)[b]		
					NA	A	DA
Li	0,85	60	acetate	5,5	8,45	12,35	18,95
		90	acetate	5,5	7,60	10,41	14,96
		180	acetate	5,5	6,84	8,64	11,59
Na	1,50	30	phosphate	4,6	8,68	12,16	18,03
		50	phosphate	4,6	7,84	10,27	14,49
		50	citrate	3,7	8,02	10,54	14,87
		50	acetate	4,6	8,11	10,72	15,01
NH_4	1,95	40	acetate	4,4	8,22	10,42	14,50
K	2,50	30	acetate	4,5	8,88	11,53	16,78
		30	citrate	3,6	8,92	11,33	16,99
		30	phosphate	4,6	8,80	11,38	16,76
		40	acetate	4,5	8,09	10,25	14,75
		40	citrate	3,6	8,12	10,09	14,94
		40	phosphate	4,6	8,01	10,13	14,74

a) relative selectivity for $-SO_3H$ group, $/H^+/ = 1$ (standard value)

b) an average retention time from 5 analyses with the particular mobile phase

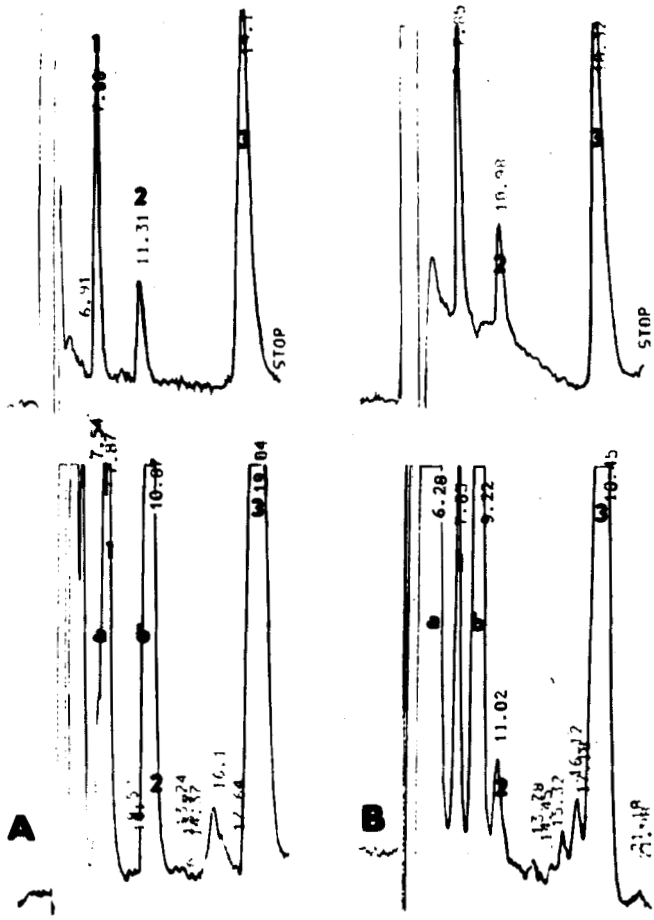

FIGURE 1. Influence of the mobile phase acidity on the elution behaviour of catecholamines and sample impurities from urine after the alumina cleanup. Mobile phase: 50 mmol/l ammonium acetate acidified with A/ 50 mmol/l /pH=3,7/, B/ 25 mmol/l /pH=4,5/ citric acid containing each 1 mmol/l EDTA and OS and 2% acetonitrile. Above: catecholamine standard with 50, 25 and 200 pmol NE, E and DA /1, 2 and 3/, bellow: urine sample, where a and b are sample impurities.

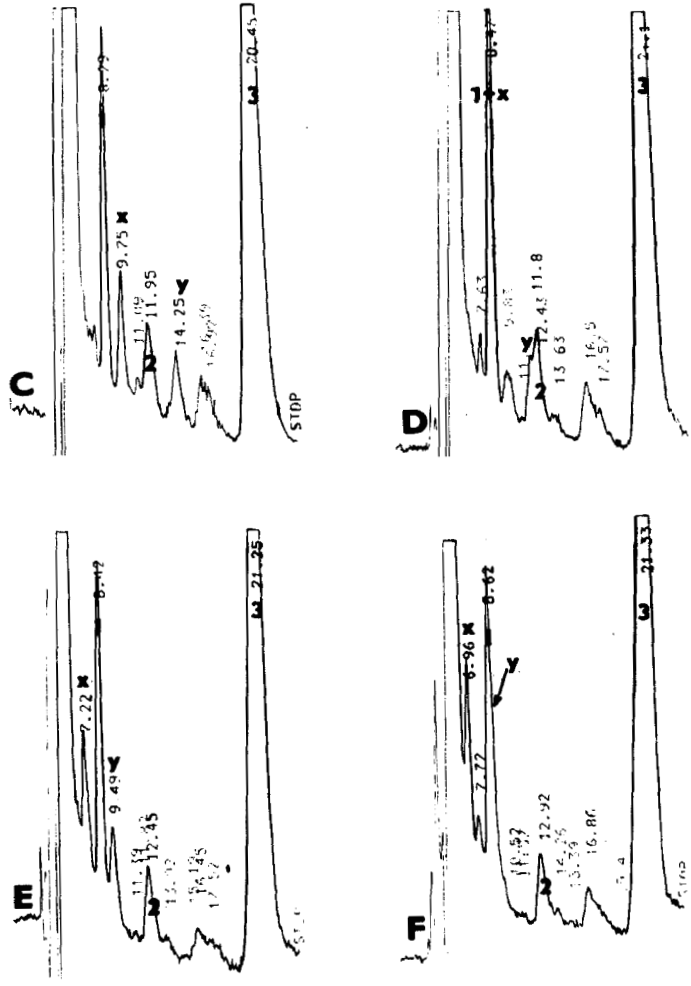

FIGURE 2. Urine sample as in figure 1 analyzed after the cleanup step in the same mobile phase as in figure 1. However, instead of citric acid the ammonium acetate was acidified with C/ 12 mmol/l acetic acid /pH=5,0/, D/ 5 mmol/l acetic acid /pH=5,15/, E/ 1 mmol per liter EDTA /pH=5,4/, F/ 0,1 mmol/l EDTA /pH=5,45/. The interfering peaks x and y are of different nature than those /a,b/ in figure 1.

/Table 1/ or with 1 mmol/l of each, EDTA and OS /Figures/. The flow rate was 0,8 or 1 ml/min, respectively.

Urine samples were subjected to alumina extraction immediately or after few days of storage at -20°C without acidification. The cleanup procedure followed that of Maycock /5/ with slight modification /6/. Briefly, micropipette tips /300 µl in volume/ were filled with about 20 mg alumina /10 mm height approximately/ fixed by means of glass-wool and 100 µl urine with 100 µl of 1 mol/l Tris buffer /pH 8,6/ was applied on the column and percolated through by suction /reduced pressure created by peristaltic pump/. After washing with 2 ml of distilled water the catecholamines were eluted by pressing 200 µl of 0,5 mol/l acetic acid through the column by means of a syringe with a luer compatible with the tip diameter. An aliquot or the whole volume of the eluate was injected into the analytical system.

RESULTS AND DISCUSSION

Dynamic cation exchange systems, created in the column sorbent by addition of a convenient IP agent into the mobile phase, behave like conventional ion exchange systems, so that the retention can be influenced, in a predictable way, by adjustement of the counterion concentration. Relative selectivities /affinities/ of various counterions toward, in this case, sulfonyl group being a strong cation exchanger, are known and given in Table 1. As we have arbitrarily chosen the concentration of ammonium ions to 40 mmol/l, the corresponding concentrations with respect to selectivity values should be for sodium and potassium 50 and 30 mmol/l and for lithium 90 mmol/l, respectively. In order to evaluate influence of counterion concentration changes, acidity of mobile phase and type of anion we prepared various solutions as apparent from Table 1. It can be seen that reten-

tion times, corresponding to the calculated concentrations of the counterions according the selectivity values, are comparable /left columns/. With one exception: the competitive influence of potassium was lower than expected and an increase to concentration of 40 nmol/l was necessary to achieve the expected comparable retention values. This finding could point to the report of Crombeen et al./1/ that K^+ cannot be used together with SDS as it forms insoluble salts with it. However, with OS as IP agent such conclusions were not drawn and, on the contrary, the use of potassium as counterion was favoured /3/. Thus, the reason for a diminished competitive effect of potassium in our system is not known.

From our results it appears that employment of ammonium cation seems to be preferable as the elution succeeds with a minimal concentration of the ion. There is an additional favourable effect because from the quantitative point of view the elution of the last peak, DA, was most complete, about 10% higher than in the other cases.

Retention times of catecholamines were practically not influenced by the type of anion used and the same was true for the accompanying interfering compounds. Their location on the chromatogram was also not affected by the type of cation. Moreover, our results confirm also the findings of Michaud et al./7/ that changes in retention times for NE, E and DA in pH range 2,8 to 3,8 were negligible with employment of C_8 column. With respect to our findings the pH range can be thus prolonged to 5,4.

Unlike the catecholamines, the accompanying interfering compounds are, in the retention, strongly influenced by even small pH changes /Figures 1 and 2/. This is in accordance with some previous findings, where a shift to lower retention times with pH increase was ob-

served with 3,4-dihydroxyphenylalanine /DOPA/ and 3,4-dihydroxyphenylacetic acid /DOPAC/, as referred /2,7/. As both these compounds can be isolated by alumina extraction in a considerable amount, i.e. with recoveries about 50% for DOPA and 12-23% for DOPAC /8,9/, it seems to be probable that impurities "a" and "b" on Fig. 1 are acidic compounds of such a nature.

By increasing pH of mobile phase to 5 and more, interfering peaks of another origin appear on the chromatogram /Fig. 2/. Their nature was not examined but it was found that their retention is dependent on pH value considerably. Best analytical performance afforded a medium with ammonium acetate /50 mmol/l/ acidified by addition of 1 mmol/l EDTA to pH 5,4 /Fig. 2E/. Under these conditions the catecholamines were separated from their interferences. Change of 0,05 unit in pH value to pH 5,45, caused by diminishing of EDTA concentration to 0,1 mmol, resulted already in coelution of NE with y interference /Fig. 2F/. By lowering pH to 5,15 the compound y is coeluted with E /Fig. 2D/ and by additional acidification to pH 5 the biogenic amines are again separated from their artefacts /Fig. 2C/.

It is realistic to expect that retention of the examined compounds will vary from column to column, from manufacture to manufacture. However, the trend in the retention behaviour should be approximately the same. An alternative use of C_{18} RP column under the same conditions in our laboratory revealed, that employment of C_8 column was advantageous. With the C_{18} column there were more difficulties in managing the interferying peaks and because of its greater retention affinity an admixture of a larger volume of organic modifier /acetonitrile, methanol/ was required. The preferable use of C_8 in comparison to C_{18} column in such or similar kind of analysis was reported previously /7,10,11/. Simple ammonium ace-

tate system was announced to be a better alternative to often complicated and expensive buffers /4/, too. Our findings seem to support such recommendations as the most simple cleanup procedure and the relatively low efficient C_8 column with 10 µm particle size afforded satisfactory results toward urine catecholamine analysis.

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