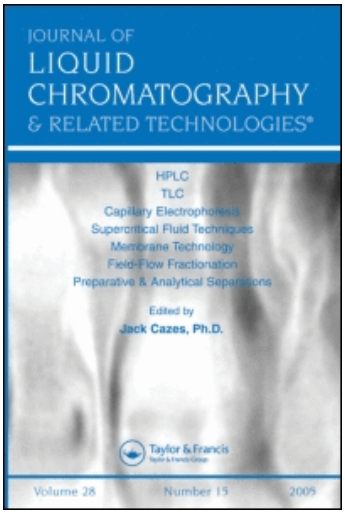

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APPLICABILITY OF REVERSED-PHASE BASE-DEACTIVATED COLUMNS FOR SYSTEMATIC TOXICOLOGICAL ANALYSIS

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ABSTRACT

The chromatographic properties of seven reversed-phase columns from different manufacturers, specially prepared for analysis of basic drugs, were investigated. Three test mixtures were used: Neutral (1-nitroalkanes), acidic (salicylamide and four barbiturates) and basic (diphenhydramine, imipramine, amitriptyline, fluphenazine and thioridazine). The mixtures were eluted with three mobile phases, consisting of acetonitrile-water, acetonitrile-phosphate buffer and acetonitrile-triethylammonium phosphate buffer. The concentration of acetonitrile, pH and molarity of buffers were identical.
The neutral and acidic drugs were separated in all mobile phases. The addition of buffer or amine to the mobile phase exerted virtually no influence on the chromatographic behavior of these compounds.
Basic drugs were not eluted in acetonitrile-water mixture. These drugs were eluted and separated fairly well in acetonitrile-phosphate buffer; the application of triethylammonium phosphate buffer was associated with faster elution of basic drugs and narrower peaks on all examined columns.
The results indicate, that the investigated base-deactivated columns - with one exception - may be used for general toxicolo-

gical screening. The silanol effects were negligible, but present in all examined columns. Therefore, although it is possible to separate basic compounds on these column in mobile phase consisting of acetonitrile and acidic buffer, the use of amine modifier is still advisable.

INTRODUCTION

The forensic toxicologist, running systematic search for an unknown harmful substance in biological sample never knows what he is looking for but has to consider possibly broadest spectrum of substances, which may be potentially involved. This requirement determines the methodology in toxicological screening; in the case of organic poisons only chromatographic methods of analysis allow to separate, identify and quantify a large number of relevant substances in one analytical run (1). The establishing of interlaboratory databases comprising several thousands of poisonous substances (2,3) was possible after standardization of thin-layer and gas chromatography. The standardization of HPLC data appeared particularly difficult, due to large differences in selectivities of nominally identical, but commercially different packing materials (4-8). In the case of mostly used reversed-phase column packings these differences have been attributed to the interaction between the uncapped silanols and the analytes, particularly those of basic character (9 - 12). Sample-silanol interactions, commonly known as "silanol effects", may be reduced by modification of mobile phase composition parameters, like pH, ionic strength of the buffer, or addition of amine modifiers (13-17). The second approach concerns the use of specially prepared column packing, showing minimum silanol activity. This has been achieved by the combination of several procedures:

- purification of silica support and elimination of trace metals,
- use of type B silica, showing more homogenous distribution of silanol groups (18-20),

- use of silane of shorter chain (i.e.octyl instead of octadecyl (19,20),
- deactivation of free silanol groups through various (often not precised) "endcapping" procedures.

In the recent review several columns of this type have been mentioned (21), indicating the introduction of a new generation of base-deactivated columns.

The purpose of this paper was to test the applicability of base-deactivated columns for analysis of various classes of compounds, which may be found in the course of general toxicological screening. Also, various elution conditions were applied in order to investigate the residual silanol activity of the columns.

The selection of the test mixtures was based on our previous studies (22, 23) and took into consideration the following points:

- assessing the efficiency of columns in relation to neutral, acidic and basic substances,
- resolution of selected, close-eluting compounds,
- elution of all compounds in reasonable time.

MATERIALS

Test Mixtures

The neutral mixture consisted of nitromethane, nitroethane, 1-nitropropane, 1-nitrobutane and 1-nitropentane (all supplied by Fluka AG, Buchs, Switzerland) dissolved in methanol to the concentration of 100 µl/ml each.

The acidic mixture consisted of salicylamide, brallobarbital, pentobarbital, secobarbital and thiopental dissolved on methanol to the concentration of 100 µg/ml each.

The basic mixture contained diphenhydramine, imipramine, amitriptyline, fluphenazine and thioridazine dissolved in methanol to the concentration of 100 µg/ml each.

Columns

Following columns were used for the study:

1.) SUPERSPHER RP-18, 125 x 4 mm, grain size 5 μm (E.Merck AG, Darmstadt, Germany), fully endcapped. This column packing was used in all our previous studies (7, 8, 22, 23) and served as a reference.

2.) TECHSPHERE ODS-BDS, 100 x 4.6 mm, grain size 5 μm (HPLC Technology Ltd, Macclesfield, UK). According to the literature (21) this column was specially developed for basic compounds, had 14% carbon-monolayer coverage and no modifier were needed for analysis.

3.) NUCLEOSIL 100-5 C18 AB, 125 x 4 mm, grain size 5 μm (Macherey Nagel GmbH, Düren, Germany) was specially developed for acidic and basic compounds. The manufacturer stated, that the separation of some basic drugs was possible also in acetonitrile-water mixtures.

4.) ENCAPHARM RP18-TS, 120 x 4.6 mm, grain size 5 μm (Dr.I.Molnar, Berlin, Germany). According to the information of manufacturer the packing had impurities level below 0.01%, very low metal content, was optimally silanized and showed homogenous distribution of silanol groups.

5.) LICHROSPHER 60 RP-select B (octyl), 125 x 4 mm, grain size 5 μm (E.Merck AG, Darmstadt, Germany) was specially developed for basic compounds and guaranteed for reproducibility.

6.) SYNCHROPAK RP-SCD, 100 x 4.6 mm, grain size 5 μm (SYNCHROM Inc., Lafayette, USA). This packing was prepared with type B silica and specially deactivated. SCD stands for "short chain deactivated"; the length of silane chain was not stated. The use of amine modifier of mobile phase had no influence on the retention time of basic drugs on this column (19).

7.) INERTSIL ODS-2, 125 x 4.6 mm, grain size μm (GL Sciences Inc., Tokyo, Japan). This packing was declared as prepared from practically metal-free, very pure silica (impurities below 0.001%) and was completely endcapped.

METHODS

HPLC Instrumentation

The HPLC system consisting of Series 1050 Ternary Pump, Series 1050 Autosampler (both from Hewlett Packard, Waldbronn, Germany) and Series 900 Diode Array Detector with Series 900 Plotter (both from Waters, Eschborn, Germany) was used.

HPLC Conditions

Three test mixtures were analyzed in duplicate on all columns in the following mobile phases (in that order):

1 - acetonitrile-water (30:70)

2 - acetonitrile-phosphate buffer 25 mM, pH 3.0 (30:70)

3 - acetonitrile-triethylammonium phosphate buffer 25 mM, pH 3.0 (30:70). This buffer was supplied by Fluka AG, Buchs, Switzerland.

The mobile phase flow rates for columns 1, 3, 4, 5 and 7 were 1.0 ml/min, and 0.8 ml/min for columns 2 and 6. The injection volumes used were 10 or 20 µl. The detector was set at 220 nm (pilot signal). The identity of each compound was checked by means of post-run UV-spectrum analysis of peaks.

Calculations

The capacity factors (k') were calculated from the dead time t_o and the retention time t_r by the equation:

$$k' = (t_r - t_o)/t_o \qquad (1)$$

The dead time was determined as first baseline disturbance after injection of 10 µl of methanol.

The effective plate count (N) values for 1-nitropentane, secobarbital and fluphenazine were calculated according to the formula:

$$N = 5.545\ (t_r - t_o)^2/W \qquad (2)$$

where W = peak width at the half height

The resolution (Rs) of salicylamide/brallobarbital and imipramine/amitriptyline was calculated by the equation:

$$Rs = W_1 - W_2\ /\ t_{r1} - t_{r2} \qquad (3)$$

where W_1 and W_2 = peak widths at the half height, and t_{r1} and t_{r2} = retention times of separated drugs.
The assymmetry factors (As) for fluphenazine were calculated by dropping a perpendicular from the peak maximum and measuring the distance from this line to the leading edge (a) and the trailing edge (b) at the 10% peak height by the formula:

$$As = 100\ (a/b) \qquad (4)$$

RESULTS AND DISCUSSION

Tables 1 - 7 show the chromatographic behavior of analyzed substances on all columns. In general, nitroalkanes were eluted as well separated, symmetrical peaks on all columns and in all mobile phases. The selectivities of the packings toward 1-nitroalkanes showed large variability; the TECHSPHERE and SYNCHROPAK columns were the fastest ones, whereas the capacity factors for ENCAPHARM and SUPERSPHER columns were twice as large. The efficiency of the columns, measured for 1-nitropentane, was distinctly lower in the mobile phase 1 for LICHROSPHER RP-select B and SUPERSPHER RP 18 columns. These columns showed the highest plate count numbers. The efficiency of TECHSPHERE column showed an exactly opposite trend. In the case of other columns no definite influence of mobile phase composition on the efficiency towards 1-nitropentane was observed. The mixture of acidic drugs has been succesfully separated in all mobile phases on all columns with exception of TECHSPHERE. Salicylamide and brallobarbital were eluted on TECHSPHERE column as one peak. The use of buffer instead of water in mobile phase has improved the efficiency and peak shapes of acidic drugs for SUPERSPHER column and LICHROSPHER select B. On four other columns the acidics drugs were eluted in acetonitrile-water phase as symmetrical, well separated peaks, and the use of buffer brought slight or no improvement. The use of amine modifier has exerted virtually no influence on the behavior of acidic drugs. In this point our observations are different than those of Freiser et

TABLE 1

SUPERSPHER RP-18 Column: Examined Parameters of Test Compounds in Three Mobile Phases.

	k' in mobile phase:		
	1	2	3
Nitromethane	0.97	0.99	1.03
Nitroethane	1.93	2.00	1.98
1-Nitropropane	4.15	4.18	4.18
1-Nitrobutane	9.33	9.27	9.18
1-Nitropentane	21.29	21.03	20.99
Salicylamide	1.65	1.72	1.75
Brallobarbital	2.85	3.24	3.33
Pentobarbital	4.75	6.84	7.22
Secobarbital	6.64	9.92	10.50
Thiopental	9.11	17.55	17.18
Diphenhydramine	-	4.54	3.70
Imipramine	-	10.70	8.71
Amitriptyline	-	13.27	10.77
Fluphenazine	-	21.63	21.27
Thioridazine	-	40.78	31.77
N(nitropentane)	29100	40400	43500
N(secobarbital)	6400	29800	18600
N(fluphenazine)	-	30800	33700
Rs(salic./brallo.)	1.5	3.0	2.7
Rs(imipr./amitr.)	-	2.0	2.1
As(fluphenazine)	-	40	40

TABLE 2

TECHSPHERE ODS-BDS Column: Examined Parameters of Test Compounds in Three Mobile Phases.

	k' in mobile phase:		
	1	2	3
Nitromethane	0.52	0.40	0.45
Nitroethane	1.04	1.18	1.12
1-Nitropropane	1.99	2.36	2.32
1-Nitrobutane	3.83	4.89	4.79
1-Nitropentane	7.76	10.26	9.99
Salicylamide	0.93	0.97	0.91
Brallobarbital	1.14	1.27	1.14
Pentobarbital	2.40	2.60	2.55
Secobarbital	3.27	3.50	3.40
Thiopental	5.02	4.65	4.54
Diphenhydramine	0.04	0.06	0.06
Imipramine	0.47	0.78	0.69
Amitriptyline	ND	ND	0.84
Fluphenazine	1.35	1.97	2.16
Thioridazine	1.75	2.62	2.47
N (nitropentane)	27800	17200	16300
N (secobarbital)	9200	8100	6300
N (fluphenazine)	-	-	-
Rs (salic./brallo.)	-	-	-
Rs (imipr./amitr.)	-	-	-
As (fluphenazine)	-	-	-

ND - not detected, probably eluted together with imipramine

TABLE 3

NUCLEOSIL C 18 AB Column: Examined Parameters of Test Compounds in Three Mobile Phases.

	k' in mobile phase:		
	1	2	3
Nitromethane	0.72	0.72	0.72
Nitroethane	1.45	1.54	1.45
1-Nitropropane	3.06	3.20	3.12
1-Nitrobutane	6.78	7.14	7.00
1-Nitropentane	15.47	16.24	16.10
Salicylamide	1.26	1.25	1.24
Brallobarbital	2.28	2.24	2.29
Pentobarbital	4.91	4.83	5.03
Secobarbital	7.13	6.93	7.28
Thiopental	12.38	12.35	12.70
Diphenhydramine	-	2.88	2.46
Imipramine	-	6.08	5.27
Amitriptyline	-	7.40	6.44
Fluphenazine	-	9.56	12.26
Thioridazine	-	20.10	17.40
N (nitropentane)	28500	28900	23000
N (secobarbital)	14500	11200	12200
N (fluphenazine)	-	11200	13900
Rs (salic./brallo.)	1.3	1.3	1.3
Rs (imipr./amitr.)	-	1.4	1.2
As (fluphenazine)	-	56	56

TABLE 4

ENCAPHARM RP18 Column: Examined Parameters of Test Compounds in Three Mobile Phases.

	k' in mobile phase:		
	1	2	3
Nitromethane	0.99	0.98	0.99
Nitroethane	2.09	2.08	2.21
1-Nitropropane	4.48	4.59	4.75
1-Nitrobutane	10.16	10.42	10.66
1-Nitropentane	23.78	23.95	24.67
Salicylamide	1.74	1.83	1.82
Brallobarbital	3.31	3.48	3.49
Pentobarbital	7.32	7.63	7.73
Secobarbital	10.60	11.17	11.30
Thiopental	18.41	19.47	19.64
Diphenhydramine	-	3.74	3.25
Imipramine	-	8.47	6.86
Amitriptyline	-	10.41	9.13
Fluphenazine	-	18.65	18.16
Thioridazine	-	40.51	25.95
N (nitropentane)	25400	23500	28400
N (secobarbital)	18600	17500	17900
N (fluphenazine)	-	21300	22600
Rs (salic./brallo.)	2.5	2.6	3.0
Rs (imipr./amitr.)	-	2.2	2.1
As (fluphenazine)	-	69	55

TABLE 5

RP Select B Column: Examined Parameters of Test Compounds in Three Mobile Phases.

	k' in mobile phase:		
	1	2	3
Nitromethane	1.00	0.72	0.76
Nitroethane	1.73	1.54	1.39
1-Nitropropane	3.27	3.32	2.95
1-Nitrobutane	6.44	7.18	6.16
1-Nitropentane	13.12	15.51	12.72
Salicylamide	1.67	1.43	1.25
Brallobarbital	2.47	2.59	2.17
Pentobarbital	4.52	5.30	4.28
Secobarbital	6.23	7.60	6.03
Thiopental	11.10	13.32	10.92
Diphenhydramine	-	3.68	2.17
Imipramine	-	7.86	4.55
Amitriptyline	-	9.94	5.57
Fluphenazine	-	14.20	9.07
Thioridazine	-	26.81	14.14
N (nitropentane)	44900	69700	66500
N (secobarbital)	31600	44700	38500
N (fluphenazine)	-	24600	25900
Rs (salic./brallo.)	1.5	1.9	2.0
Rs (imipr./amitr.)	-	1.6	1.6
As (fluphenazine)	-	60	50

al.(19) and Hill (20), who had observed an improvement of peak symmetry and increase of plate count number for salicylic acid after addition of triethylamine to the mobile phase, using SYNCHROPAK SCD and Zorbax RX columns, respectively. The efficiency of columns, measured as effective plate count number for secobarbital was highest for LICHROSPHER Select B column and definitely lowest for TECHSPHERE ODS BDS column. For all other

TABLE 6

SYNCHROPAK RP SCD Column: Examined Parameters of Test Compounds in Three Mobile Phases.

	k' in mobile phase:		
	1	2	3
Nitromethane	0.80	0.80	0.80
Nitroethane	1.32	1.39	1.40
1-Nitropropane	2.32	2.40	2.40
1-Nitrobutane	4.09	4.35	4.21
1-Nitropentane	7.40	7.95	7.87
Salicylamide	1.33	1.44	1.40
Brallobarbital	2.16	2.37	2.34
Pentobarbital	3.44	3.77	3.70
Secobarbital	4.57	5.10	4.98
Thiopental	7.06	7.94	7.75
Diphenhydramine	-	3.29	2.62
Imipramine	-	5.97	4.71
Amitriptyline	-	6.86	7.75
Fluphenazine	-	8.27	7.75
Thioridazine	-	15.49	12.20
N (nitropentane)	19900	20600	22200
N (secobarbital)	29300	19800	19000
N (fluphenazine)	-	29300	32200
Rs (salic./brallo.)	2.2	1.8	1.9
Rs (imipr./amitr.)	-	1.1	1.3
As (fluphenazine)	-	90	90

columns the plate count number for secobarbital was in the same order.

Therefore, five base-deactivated columns: i.e. NUCLEOSIL C18-AB, ENCAPHARM RP-18, LICHROSPHER select-B, SYNCHROPAK SCD and INERTSIL ODS-2 are suitable for analysis of acidic drugs in acetonitrile-water mixtures. For SUPERSPHER RP-18 column the use of buffered phase was advisable. The TECHSPHERE ODS-BDS column showed unacceptable low selectivity in applied conditions.

TABLE 7

INERTSIL ODS-2 Column: Examined Parameters of Test Compounds in Three Mobile Phases.

	k' in mobile phase:		
	1	2	3
Nitromethane	0.72	0.69	0.70
Nitroethane	1.42	1.46	1.48
1-Nitropropane	3.08	3.23	3.24
1-Nitrobutane	6.90	7.30	7.30
1-Nitropentane	15.53	16.57	16.87
Salicylamide	1.28	1.30	1.33
Brallobarbital	2.46	2.51	2.60
Pentobarbital	5.40	5.46	5.74
Secobarbital	7.87	7.92	8.35
Thiopental	13.63	13.78	14.34
Diphenhydramine	-	2.80	2.37
Imipramine	-	6.46	5.45
Amitriptyline	-	7.92	6.71
Fluphenazine	-	13.73	14.06
Thioridazine	-	22.97	19.35
N (nitropentane)	21400	17900	21400
N (secobarbital)	16000	14800	15100
N (fluphenazine)	-	25900	25300
Rs (salic./brallo.)	2.0	2.2	2.2
Rs (imipr./amitr.)	-	1.9	2.1
As (fluphenazine)	-	50	86

None of the columns could separate and elute the mixture of basic drugs in acetonitrile-water mobile phase. The drugs were eluted very fast and not separated on the TECHSPHERE column; in the case of other columns, no peaks were observed during 60 min.

*Figs.*1 - 7 show the chromatograms of basic mixture, separated in acetonitrile-phosphate buffer and acetonitrile-triethylammonium phosphate buffer on each column. The use of acetonitrile-phosphate buffer, pH 3.0, lead to elution of all basic drugs.

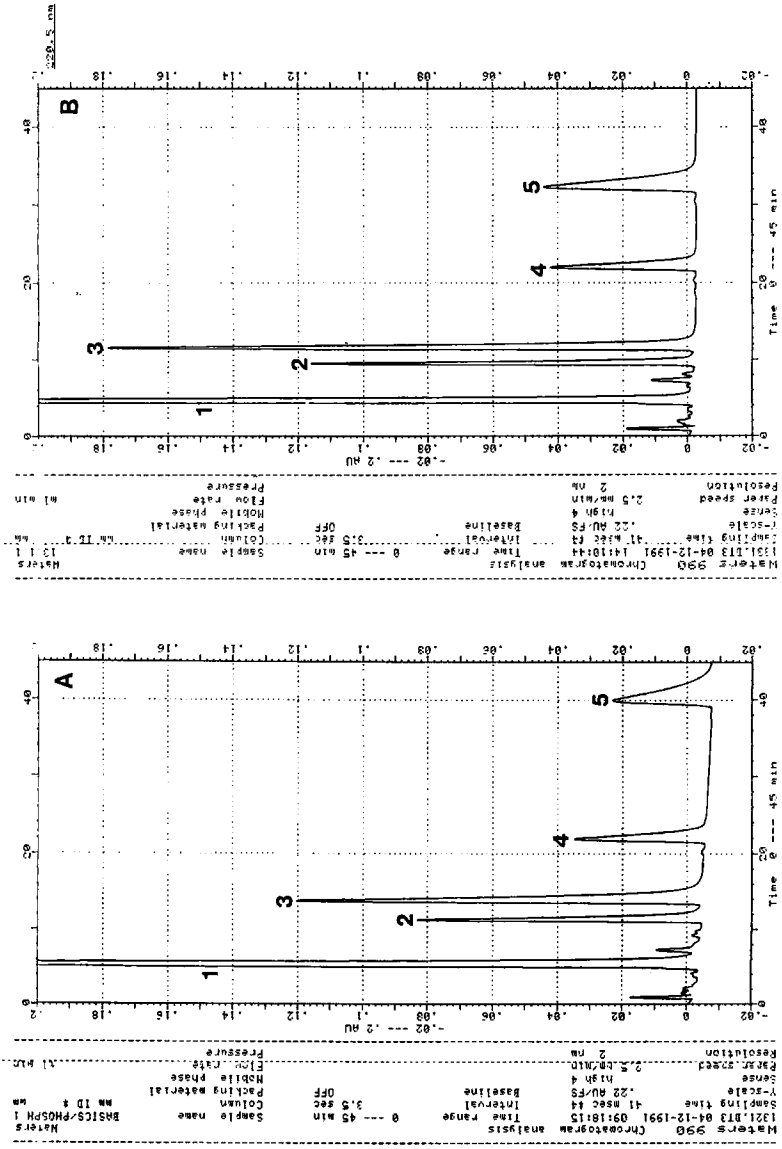


Fig.1: The chromatograms of basic drugs mixture in acetonitrile-phosphate buffer (A) and triethylammonium phosphate buffer (B). 1= diphenhydramine, 2= imipramine, 3= amitriptyline, 4= fluphenazine, 5= thioridazine. SUPERSPHER RP-18 column.

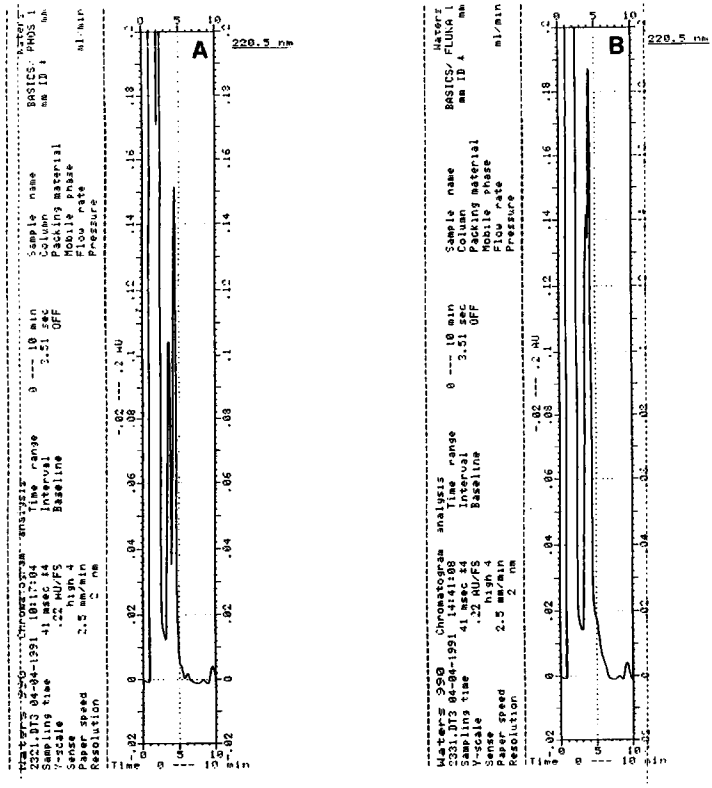
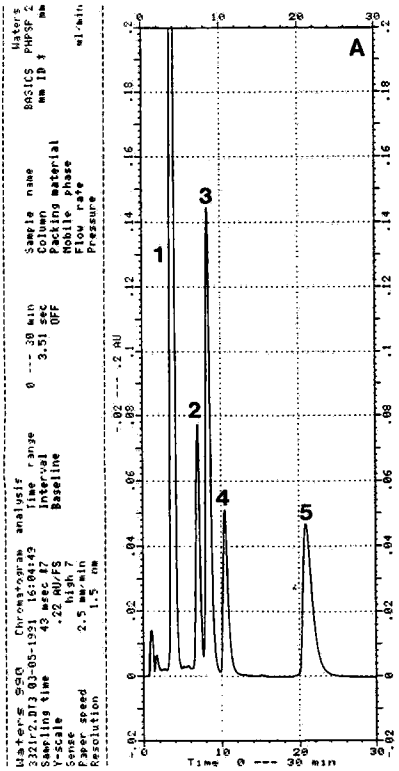


Fig.2: The chromatogram of basic drugs mixture in acetonitrile-phosphate buffer (A) and triethylammonium phosphate buffer (B). TECHSPHERE ODS-BDS column. The separation was not achieved.

Obviously, the application of acidic pH suppressed the ionization of acidic silanol groups, allowing the elution of drugs. In the case of TECHSPHERE column the drugs were eluted very fast and not separated. The other columns have separated all basic compounds. The fastest elution times were observed for SYNCHROPAK and NUCLEOSIL columns; the ENCAPHARM and SUPERSPHER column were the most selective ones. The use of mobile phase consisting of aceto-



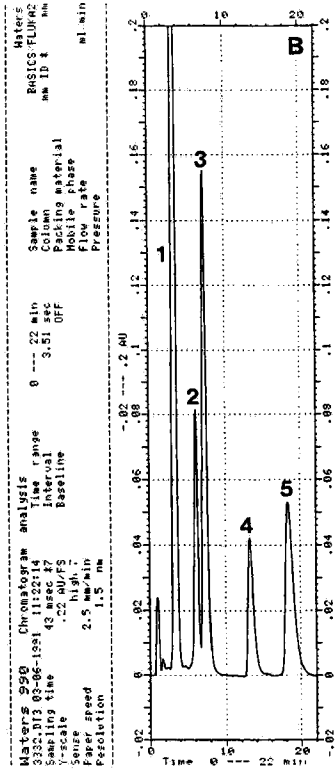


Fig.3: NUCLEOSIL C-18 AB column. Other explanations see Fig.1.

nitrile and triethylammonium phosphate was associated with shortening of elution time of basic drugs on all columns but TECHSPHERE. Only in the case of fluphenazine this effect was negligible. Most distinct reduction of retention time of basic drugs as influence of amine modifier was noted in the case of LICHROSPHER Select B and ENCAPHARM RP-18 columns (reduction of Rt to ca. 60% of value without amine). In the case of other columns, the ca.20% reduction of retention time of basic drugs was observed. (Fig. 8) Also, the efficiency of columns, measured as N(fluphenazine), was improved for all columns except INERTSIL,

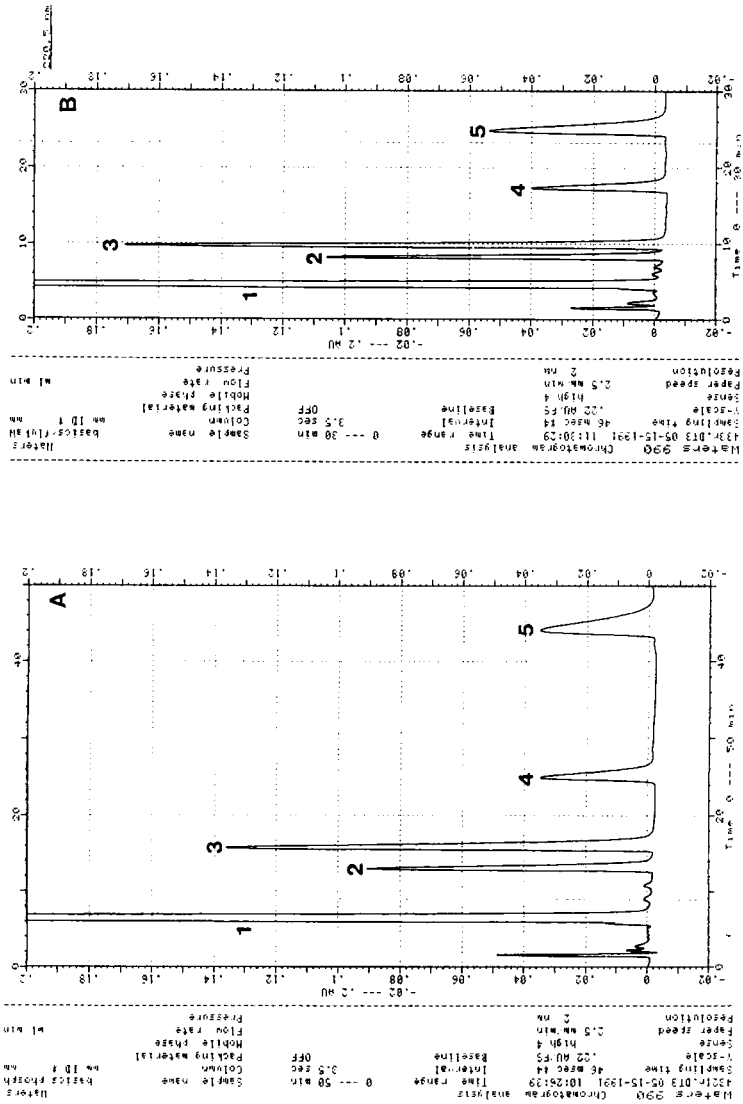


Fig.4: ENCAPHARM RP18 column. Other explanations see Fig.1.

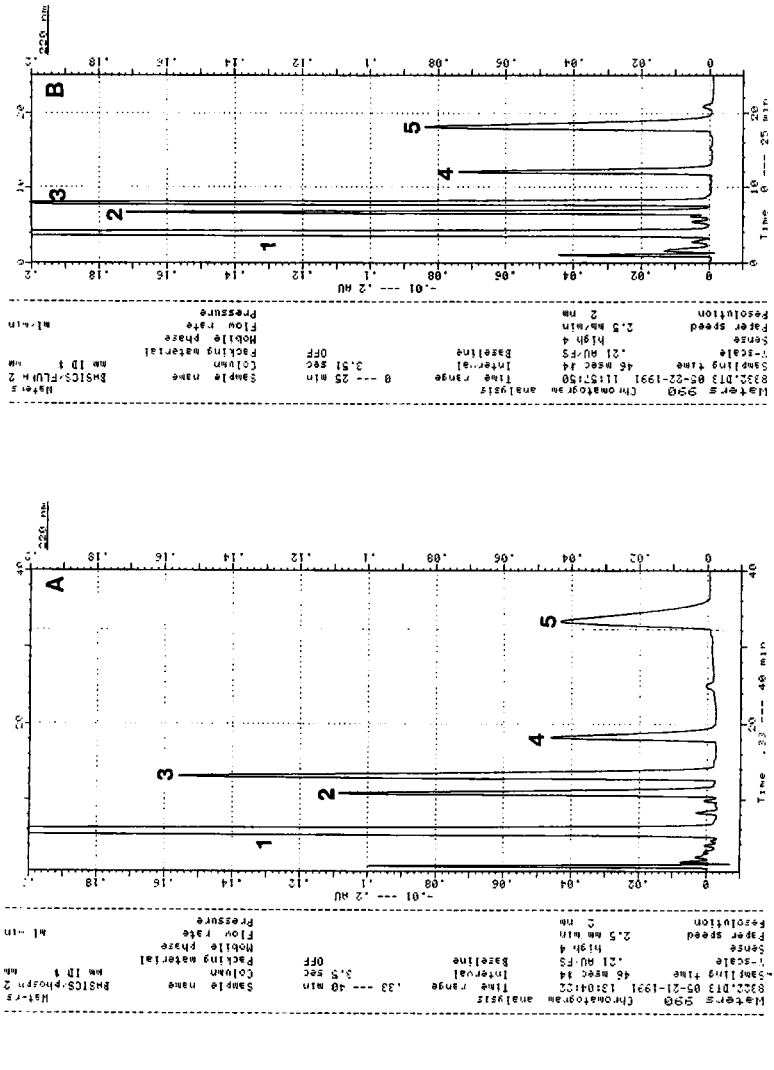


Fig.5: LICHROSPHER RP-select B column. Other explanations see Fig.1.

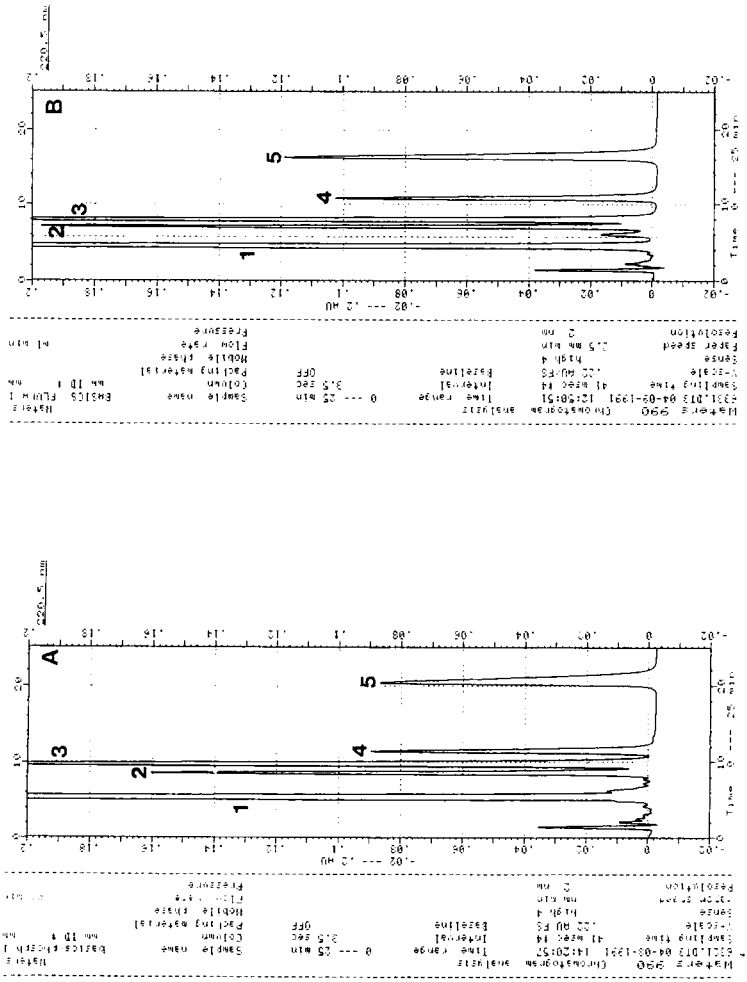


Fig.6: SYNCHROPAK RP-SCD column. Other explanations see Fig.1.

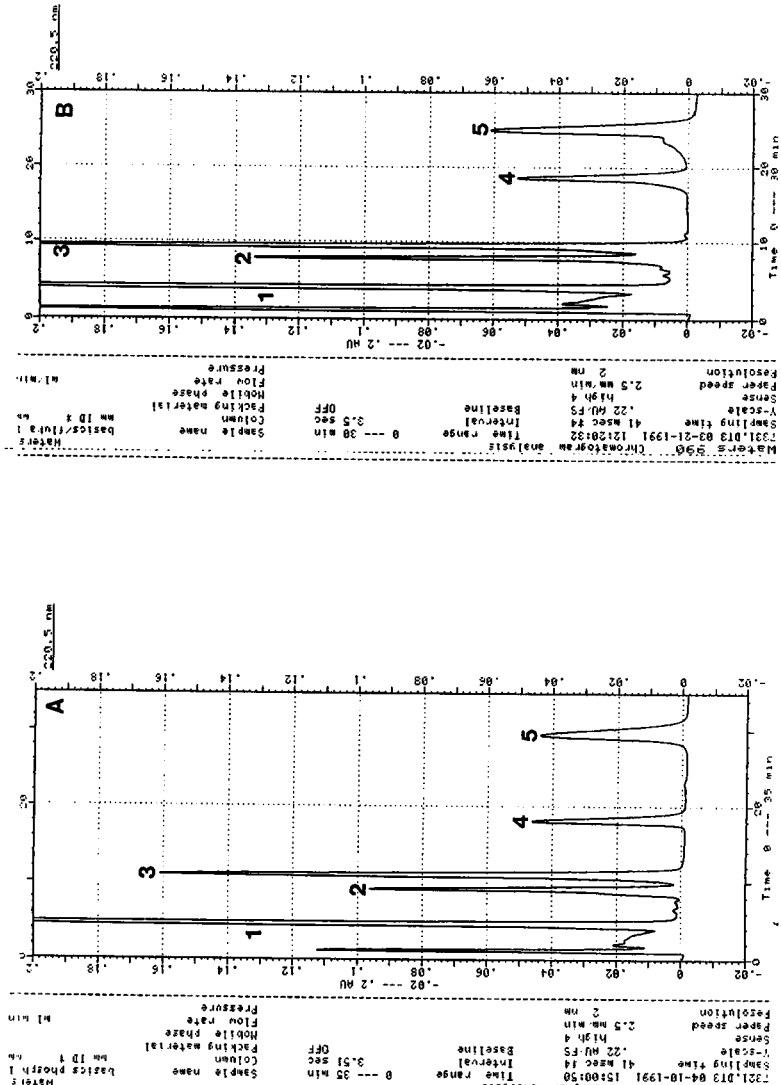
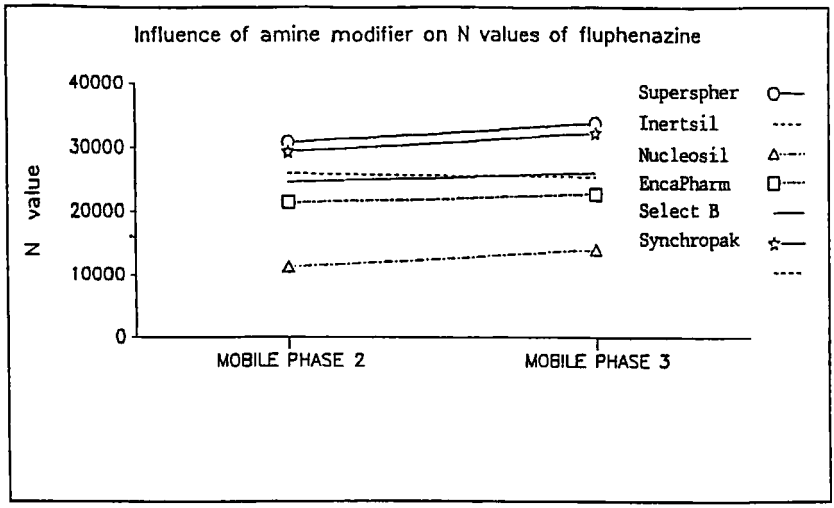


Fig.7: INERTSIL ODS-2 column. Other explanations see Fig.1.



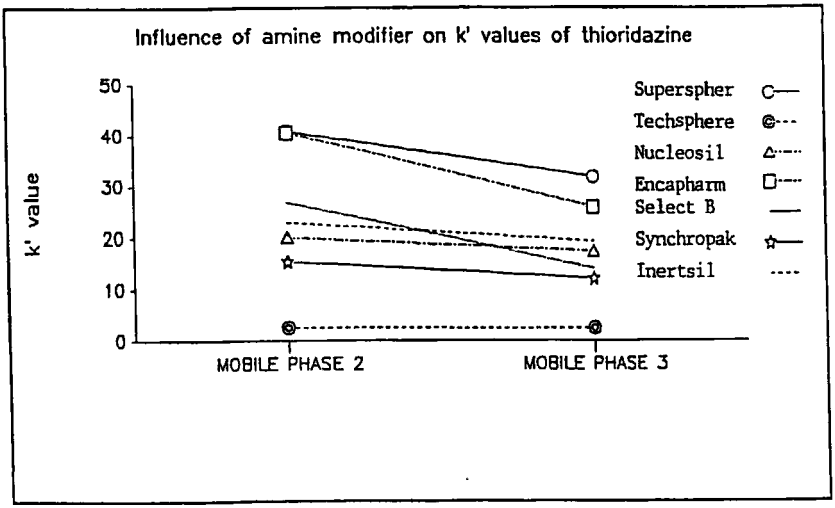


Fig.8: Efficiency ($N_{fluphenazine}$) and selectivity ($k'_{thioridazine}$) of examined columns in acetonitrile-phosphate buffer (mob. phase 2) and in acetonitrile-triethylammonium phosphate buffer (mob.phase 3)

using the mobile phase with amine modifier. This may suggest, that all columns have still some free silanol groups.

The resolution of amitriptyline and imipramine, as well as peak symmetry of fluphenazine, were not affected by the use of triethylammonium phosphate buffer.

In conclusion it may be stated, that the silanol effects in base-deactivated columns are negligible. In principle it is possible to run an analysis of all kind of drugs, including basic substances, in a mobile phase consisting of organic modifier and acidic buffer. On the other hand, the use of amine modifier brought an improvement of chromatographic behavior of basic drugs. Therefore, it still seems advisable to use a mobile phase containing an amine modifier in the case of systematic toxicological screening.

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