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HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY ON AMINO-BONDED SILICA GEL: APPLICATION TO BARBITURATES AND STEROIDS*

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SUMMARY

The retention of some barbiturates and steroids on amino-bonded silica gel pre-coated plates was examined by employing high-performance thin-layer chromatography and compared with that on silica gel pre-coated plates, with respect to the pure organic solvent used as the eluent. Aqueous and non-aqueous binary mixtures were also tested as eluents. The retentions on the two types of plates were generally different. For the pure organic solvents, the relative solvent strength on NH_2-modified plates did not follow an order resembling that reported for other polar supports, such as alumina. The subdivision of the solvents in several classes, postulated for silica support, may explain some differences in chromatographic behaviour with respect to the eluent and to the compounds examined.

INTRODUCTION

In the past 10 years there has been wide interest in reversed-phase column and thin-layer high-performance liquid chromatography (RP-HPLC and RP-HPTLC) based on the use of non-polar bonded-phase supports, such as C_{18} and C_8 chemically modified silica. Several papers have reported the characteristics and the possibility of using these types of TLC plates[1–3] and the number of separations obtained by RP-TLC is increasing[4]. More recently, polar-bonded phase supports, with amino, cyano or diol as the functional groups chemically bonded to the silica, have been successfully employed in column HPLC, and aminopropyl-bonded silica gel plates for HPTLC, pre-coated with the same type of packing material as used for HPLC, have become commercially available. However, there have been very few TLC studies

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using these commercial or home-made NH_2-modified plates[5–7]. In previous papers we reported the conditions for use and comparisons of various types of commercially available thin-layer plates pre-coated with silica gel[8,9] or with chemically modified silica gel, such as C_{18}, C_{12} and C_8[2]. The aim of this work was to investigate the behaviour of these NH_2-modified silica gel pre-coated plates by employing non-aqueous and aqueous mobile phases ("normal"- and "reversed"-phase separations, respectively). Using thin-layer chromatography, many eluting systems can be tested inexpensively in a very short time and the resulting information may be useful in column chromatography, as there is growing interest in the use of NH_2-bonded phase supports in HPLC, but few data are available on how the variation of mobile phase composition can be used to increase separation selectivity. The interpretation of the mechanism of sample retention is still controversial[10–12].

Barbituric acid derivatives and some steroids were chosen as test solutes. These compounds offer good possibilities for the study of specific polar and hydrogen bonding interactions with the amino groups of the modified silica gel support. For the sake of comparison, the retentions of these compounds on non-modified silica gel pre-coated plates for HPTLC, employing pure organic solvents as eluents, are also reported.

EXPERIMENTAL

Compounds

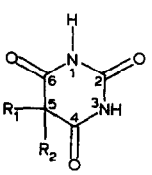
Table I lists the barbiturates and Fig. 1 shows the steroids examined; 0.1–1% solutions in methanol for the barbiturates and in methanol–chloroform for the steroids were employed.

Thin-layer chromatography

Plates. Two types of commercially available pre-coated plates for HPTLC, 5 × 5 cm and containing a fluorescent indicator, were used: HPTLC NH_2 F_{254} (Cat.

TABLE I

BARBITURATES TESTED



No.	*Trivial name*	R_1, R_2
1	Barbital	5,5-Diethyl
2	Phenobarbital	5-Phenyl-5-ethyl
3	Diallylbarbital	5,5-Diallyl
4	Cyclobarbital	5-(1-Cyclohexenyl)-5-ethyl
5	Itobarbital	5-Allyl-5-isobutyl
6	Heptabarbital	5-(1-Cycloheptenyl)-5-ethyl
7	Amobarbital	5-Ethyl-5-(3-methylbutyl)
8	Secobarbital	5-Allyl-5-(1-methylbutyl)

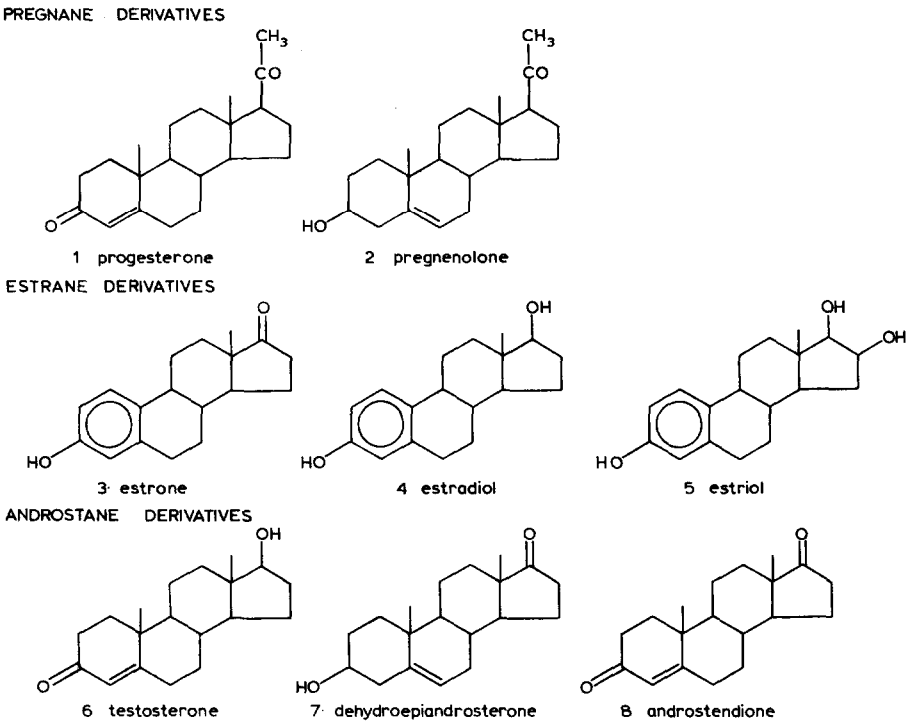


Fig. 1. Steroids tested.

No. 15647) and silica gel 60 F_{254} (Cat. No. 5628), both from E. Merck (Darmstadt, F.R.G). No pre-treatment of the plates was performed.

Eluents. The solvents and the water used were all of HPLC grade and obtained from Farmitalia-Carlo Erba (Milan, Italy). The solvents and abbreviations used in Fig. 2 are as follows: diethyl ether (EE), chloroform (CH), methylene chloride (MC), ethylene chloride (EC), tetrahydrofuran (THF), acetone (A), ethyl acetate (EA), nitromethane (NM), acetonitrile (AC), isopropanol (i-PrOH), ethanol (EtOH) and methanol (MeOH). Aqueous (a) and non-aqueous (b) eluents were also employed: (a) mixtures containing 5–90% (v/v) of an organic modifier and water (or 0.1–1 *M* acetic acid); the organic modifiers were acetonitrile, methanol and ethanol; (b) chloroform–methanol, cyclohexane–methanol or *n*-hexane–ethanol, in different ratios.

Spotting and development. To achieve high resolution, the spot must be very small[13] and in this work it was usually about 2–3 mm^2. Portions of 0.2–0.3 μl of solutions of barbiturates and steroids were applied 1 cm apart from the lower edge of the plate using a thin capillary pointed at the tip. The development was performed in a glass jar (10.5 cm high × 6.5 × 6.5 cm) equipped with a ground-glass stopper. Volumes of 12 ml of eluent were placed in the glass jar 20 min prior to insertion of the plates. Ascending development was carried out at room temperature for 3.5 cm.

Detection. After development, for the barbiturates the wet plates were exposed to an ammonia atmosphere for 5 min, whereas for the steroids the plates were dried.

The spots were located under ultraviolet light by quenching of fluorescence at 254 nm. A Perkin-Elmer Model 650-10s fluorimeter, equipped for densitometric measurements, in the reflectance mode, was used to measure directly the barbiturate and steroid spots after the separation on the chromatographic plates. The plates were scanned in a direction parallel to that of the solvent flow.

RESULTS AND DISCUSSION

For comparable experimental conditions and employing the various eluents investigated, the following parameters were kept constant: solvent volume in the developing tank, time prior to the insertion of the plates, distance of the starting line from the bottom and distance of development. All the experiments were performed at room temperature. Each experiment was repeated three to five times and the R_F values were averaged.

As solutes, some 5,5-disubstituted barbituric acid derivatives and some steroids, including estrane, androstane and pregnane derivatives, listed in Table I and Fig. 1, respectively, were tested. Barbiturates, differing in aliphatic or aromatic side-chains and steroids, with different configurations, degree of unsaturation, chemical functions and side-chains, were chosen.

Various solvents (see Experimental), with increasing polarity and differing in the proton-donor solubility parameter[14], chosen from those most commonly used in HPLC, were investigated as eluents, either pure or in binary mixtures (aqueous and non-aqueous mixtures).

Eluents

Pure organic solvents. Tables II and III show the R_F values of the barbiturates

TABLE II

R_F VALUES OF BARBITURATES ON (a) HPTLC NH_2-BONDED AND (b) HPTLC SILICA GEL PRE-COATED PLATES

Eluent	*Barbiturate**															
	1		*2*		*3*		*4*		*5*		*6*		*7*		*8*	
	a	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*
Diethyl ether	0.02	0.76	0.00	0.76	0.02	0.81	0.03	0.77	0.05	0.85	0.01	0.80	0.04	0.82	0.05	0.82
Chloroform	0.02	0.02	0.00	0.04	0.00	0.06	0.00	0.04	0.02	0.09	0.01	0.07	0.03	0.08	0.03	0.07
Methylene chloride	0.00	0.00	0.00	0.04	0.00	0.07	0.00	0.10	0.00	0.11	0.00	0.04	0.00	0.07	0.00	0.10
Ethylene chloride	0.00	0.05	0.00	0.05	0.00	0.03	0.00	0.03	0.00	0.09	0.00	0.03	0.00	0.06	0.00	0.08
Tetrahydrofuran	0.35	0.90	0.18	0.91	0.30	0.94	0.31	0.90	0.48	0.95	0.33	0.89	0.46	0.88	0.49	0.88
Acetone	0.48	0.88	0.30	0.89	0.44	0.89	0.44	0.90	0.55	0.92	0.44	0.91	0.54	0.91	0.57	0.90
Ethyl acetate	0.06	0.84	0.02	0.86	0.05	0.88	0.04	0.87	0.12	0.90	0.04	0.88	0.10	0.89	0.11	0.90
Nitromethane	0.10	0.56	0.03	0.67	0.07	0.68	0.05	0.66	0.13	0.74	0.08	0.68	0.19	0.72	0.13	0.74
Acetonitrile	0.07	0.83	0.02	0.92	0.07	0.89	0.04	0.93	0.15	0.95	0.07	0.91	0.10	0.93	0.10	0.93
Isopropanol	0.18	0.84	0.05	0.84	0.11	0.87	0.12	0.86	0.30	0.88	0.17	0.84	0.20	0.84	0.27	0.84
Ethanol	0.31	0.83	0.14	0.87	0.26	0.82	0.24	0.82	0.37	0.87	0.24	0.82	0.36	0.83	0.40	0.79
Methanol	0.55	0.78	0.42	0.84	0.51	0.80	0.50	0.82	0.55	0.86	0.50	0.83	0.60	0.84	0.59	0.78

* Numbering as in Table I.

TABLE III

R_F VALUES OF STEROIDS ON (a) HPTLC NH_2-BONDED AND (b) HPTLC SILICA GEL PRE-COATED PLATES

Eluent	*Steroid**															
	1		*2*		*3*		*4*		*5*		*6*		*7*		*8*	
	a	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*
Diethyl ether	0.56	0.59	–	–	0.32	0.70	0.20	0.59	0.02	0.11	0.30	0.44	0.40	0.53	0.51	0.51
Chloroform	0.76	0.18	0.48	0.23	0.15	0.12	0.08	0.03	0.00	0.00	0.43	0.08	0.48	0.28	0.77	0.18
Methylene chloride	0.69	0.15	0.28	0.21	0.13	0.19	0.08	0.05	0.00	0.00	0.33	0.08	0.47	0.24	0.68	0.16
Ethylene chloride	0.60	0.11	0.30	0.15	0.12	0.15	0.05	0.05	0.00	0.00	0.25	0.05	0.32	0.14	0.57	0.10
Tetrahydrofuran	0.84	0.84	0.76	0.74	0.73	0.83	0.60	0.75	0.38	0.75	0.78	0.80	0.68	0.70	0.76	0.72
Acetone	0.84	0.79	0.75	0.78	0.73	0.79	0.65	0.76	0.43	0.72	0.78	0.74	0.75	0.71	0.80	0.80
Ethyl acetate	0.78	0.78	–	–	0.53	0.81	0.42	0.75	0.06	0.42	0.59	0.67	0.60	0.68	0.75	0.73
Nitromethane	0.84	0.69	–	–	0.35	0.75	0.27	0.56	0.03	0.03	0.57	0.55	0.53	0.50	0.75	0.68
Acetonitrile	0.86	0.92	–	–	0.65	0.90	0.59	0.90	0.10	0.81	0.75	0.84	0.70	0.86	0.82	0.89
Isopropanol	0.80	0.76	0.70	0.68	0.64	0.73	0.70	0.82	0.58	0.73	0.78	0.75	0.70	0.72	0.78	0.72
Ethanol	0.86	0.80	0.78	0.80	0.71	0.73	0.74	0.82	0.71	0.78	0.84	0.79	0.71	0.66	0.83	0.80
Methanol	0.86	0.83	0.68	0.62	0.68	0.77	0.76	0.83	0.74	0.80	0.86	0.86	0.77	0.75	0.86	0.82

* Numbering as in Fig. 1.

and steroids, respectively, on amino-bonded and silica gel plates, obtained by employing the twelve pure organic solvents tested as eluents. The R_F values of pregnenolone (steroid 2) are not always reported, as in several eluents (diethyl ether, ethyl acetate, nitromethane and acetonitrile) it showed long tails or remained at the point of application. This behaviour seemed to be related to its solubility as it is hardly soluble in the above solvents. The R_F values of the barbiturates and of the steroids reported in Tables II and III, respectively, are plotted together in Fig. 2 in order (a) to demonstrate the different behaviour between the two classes of compounds on the NH_2-bonded support with respect to their retention on silica gel and (b) to show the trend of the relative solvent strength on NH_2-bonded pre-coated plates. In Fig. 2 the chromatograms are ordered according to increasing values of Snyder's solvent strength, ε_0[14].

From a comparison of the chromatograms on both types of plates and from the results on amino-bonded plates, it was possible to draw some conclusions about the characteristics of the latter type of support, as follows. Barbiturates were more retained on NH_2-modified than on silica plates, whereas the steroids, in relation to the solute and to the eluent, were retained differently. The retention on amino-modified plates generally differs significantly from that on silica plates, in agreement with the results of Hennion *et al.*[11] and Snyder and Schunk[12] and not with those of Hammers *et al.*[10] on NH_2 columns in HPLC.

When the succesive chromatograms in the twelve eluents, shown in Fig. 2, were compared it appeared that, on NH_2-modified plates, the sequence of retention of the test compounds in relation to the eluents employed did not increase, following an order corresponding to the increasing values of the Snyder and Schunk's eluent strength, ε_0, on alumina as adsorbent[14]. Indeed, with diethyl ether the barbiturates began to move, whereas they remained at the point of application with chloroform,

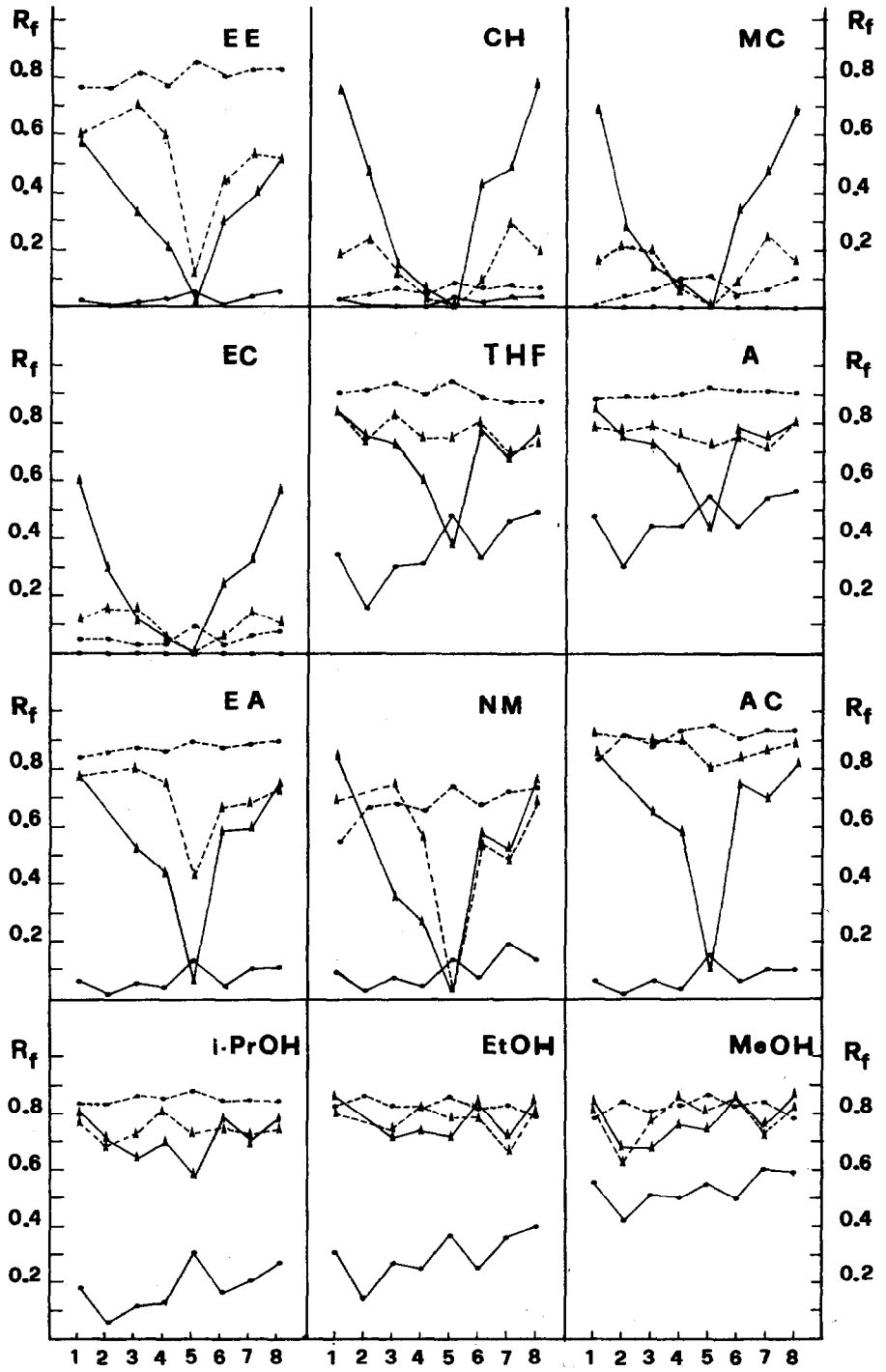


Fig. 2. R_F values of barbiturates (●) and steroids (▲) on HPTLC NH_2-bonded (solid lines) and HPTLC silica gel pre-coated plates (broken lines) as a function of the pure organic solvent used as eluent. The chromatograms are ordered according to increasing values of the Snyder solvent strength, ε_0. Designation of barbiturates as in Table I, of steroids as in Fig. 1 and of the solvents as in Experimental. Plates 5 × 5 cm; ascending development for 3.5 cm.

methylene chloride and ethylene chloride as eluents, and estrone and estradiol showed higher values in diethyl ether than in the other solvents. In addition, the R_F values of the above compounds and of estriol were higher with tetrahydrofuran or acetone than with ethyl acetate, nitromethane or acetonitrile as eluents, although acetone was considered to be less polar than nitromethane, and diethyl ether less polar than chloroform, methylene chloride or ethylene chloride. The HPTLC data suggest that the relative solvent strength on NH_2-modified plates did not follow an order resembling that for polar packings such as alumina, according to the results of Hennion *et al.*[11] and Snyder and Schunk[12] on amino-bonded silica gel columns. This result may be of particular interest for the choice of the eluent for separations on this type of packing, as mainly the solutes that may have strong interactions with the amino groups may be less retained with tetrahydrofuran or acetone than with more polar solvents as nitromethane or isopropanol.

By comparing the retentions of the two series of test compounds on both the pre-coated plates, it appeared that with respect to the eluents employed, the chromatograms may be divided into groups: (a) chloroform, methylene chloride and ethylene chloride, with retention of barbiturates on NH_2-modified plates being slightly stronger than retention on silica plates and retention of steroids on NH_2-modified plates being equal to or weaker than the retention on silica plates; (b) diethyl ether, tetrahydrofuran, acetone, ethyl acetate, nitromethane and acetonitrile, with retention of barbiturates on NH_2-modified plates being considerably stronger than the retention on silica plates and, for the steroids, retention on NH_2-modified plates being equal to or greater than the retention on silica plates; (c) isopropanol, ethanol and methanol, with retention of barbiturates on NH_2-modified plates being much greater than retention on silica plates, whereas for the steroids the retentions were almost identical. For the NH_2-modified plates, the assumption of more than one solvent class as postulated for silica supports[15,16] may explain the difference in the chromatographic behaviour of the solutes observed with the various pure organic solvents as eluents. Indeed, chloroform, methylene chloride, nitromethane and acetonitrile are included in the "inert" and diethyl ether, tetrahydrofuran, acetone and ethyl acetate in the "solvating" Rogers' groups, respectively[15]. Nitromethane and acetonitrile were found to be "non-conforming" solvents as on the basis of some parameters, they may be considered as "solvating" solvents[15]. In HPTLC on NH_2-modified plates they seemed to be more similar to the solvating solvents than to the inert solvents. The alcohols form a third group, "hydroxyl". Rogers reported that on silica gel plates the solvating solvents provided for relatively greater solute mobility than the inert solvents. This is also the case with NH_2-bonded plates. The well known classification of the solvents by Snyder and Poppe[16] also corresponds well with the HPTLC results on NH_2-modified plates: chloroform is included in class N, diethyl ether, tetrahydrofuran, acetone, ethyl acetate and acetonitrile in class P and methanol in class AB. For the NH_2-bonded plates also, the subdivision of the solvents into several classes may explain some of the differences in the chromatographic behaviour and this difference must be kept in mind when choosing the eluent for a given separation.

As regards the sequence of retention of the barbiturates and steroids on NH_2-bonded plates, it remained almost the same whatever the organic solvent used as the eluent, whereas, as it expected, all the R_F values decreased or increased. In practice, in HPTLC on amino-bonded silica gel supports, the variation of the mobile

phase composition can be used to increase the selectivity of separation (*e.g.*, the separation of estrone, estradiol and estriol is not possible with chloroform or methylene chloride, whereas it is easy with diethyl ether or tetrahydrofuran), but solvent-selectivity effects seemed generally to be less important for the separation on amino-bonded supports than on alumina or silica.

The eight steroids tested showed very different retentions on NH_2-modified plates. Hydrogen bonding certainly plays an important role; indeed, compounds with a phenolic A ring and/or alcoholic OH groups were more retained. Generally, in all the solvents tested, the retention order was: estriol > estradiol > estrone > pregnenolone > testosterone ≈ dehydroepiandrosterone > progesterone ≈ androstendione.

The retention order of barbiturates on the NH_2-modified plates is not easy to understand. It is different from that on silica gel (see Fig. 2) or RP-18 plates[2], whereas it is similar to that found by HPLC on Spheron-DEAE packing, an anion exchanger with diethylamino groups[17]. Strong interactions were postulated between the CO–NH–CO–NH–CO group of the barbituric acid and the diethylamino groups present in this column packing, probably owing to hydrogen bonding and dipole interactions. Long branched aliphatic, alicyclic and aromatic substituents at the C-5 position decreased the acidity of barbituric acid and may sterically hinder these interactions, decreasing the retention of the derivatives to different extents, depending on the type of substituent. Probably the same types of interactions predominate with amino-bonded silica gel supports, as all the barbiturates were strongly retained. Moreover, the retention order seemed to be related to the nature of the substituent groups in the C-5 position. Generally, in all the solvents tested, the compounds with an aromatic ring or both allyl substituents in the side-chains (pheno-, cyclo-, hepta- and diallyl-barbital) were more retained than those with aliphatic side-chains (barbital, ito-, amo- and seco-barbital) as on the Spheron-DEAE column.

Binary mixtures. Most of these experiments were performed with barbiturates as test compounds. To investigate the effect of the water on the R_F values various mixtures of acetonitrile, ethanol, methanol and water or 0.1–1 *M* acetic acid, in different ratios, were tested. A double front was observed with acetonitrile–water (50:50 and 70:30) and the results were considered not to be useful for comparison purposes. In ethanol–water and methanol–water mixtures, the R_F values increased, as the amount of water in the eluent increased, whereas the possibility of the separation decreased, since the difference between the lower and the higher R_F values decreased. The sequence of retention remained nearly the same. Similar results were obtained by employing as eluents mixtures of methanol–acetic acid solutions. The increase in R_F values with increasing water content of eluents was also found by Jost and Hauck[5], who investigated the chromatographic behaviour of nucleosides and nucleotides on NH_2-modified plates. We agree with the opinion of Jost and Hauck that the increase in R_F values with increasing solvent polarity cannot be explained using a reversed-phase model. The alkylamino chains of the amino-bonded support can only undergo weak hydrophobic interactions with the barbiturates; indeed, amobarbital (7) and secobarbital (8), the barbiturates least retained on NH_2-modified plates, were the most retained in HPTLC on an RP-18 support[2], where the hydrophobic interactions play an important role. A difference in retention based on anion exchange may be invoked for nucleosides and nucleotides that differ in the number

of negative charges[5]. With barbiturates, which are all weak diprotic acids, ion-exchange interactions, equal for all the compounds, may have the predominant effect while these compounds were all strongly retained, but the difference in retention seemed to be related to the nature of the side-chain substituents as found in the pure organic solvents.

For the barbiturates, the same sequence was found in mixtures of two organic solvents in different ratios (chloroform–methanol; cyclohexane–methanol; *n*-hexane–ethanol). In these cases also, as the polarity of the mixtures increased, an increase in the R_F values was observed, whereas the possibility of separation decreased. As regards the steroids, the R_F values increased greatly in mixtures of acetonitrile or methanol containing only 5% of water, whereas the possibility of separations decreased.

For the steroids the greatest difference in R_F values was found in pure organic solvents as eluents. For the barbiturates the possibility of using this amino-bonded silica gel support with a wide variety of eluting systems, such as pure organic solvents or aqueous or non-aqueous binary mixtures, was found. This possibility may be interesting if, as is often the case, barbiturates must be separated as a class from other classes of compounds.

R_F values

On NH_2-modified plates the spots appeared well defined and roundish and salt addition[5] was unnecessary. The elution time was always very short, from 4 min with chloroform, tetrahydrofuran, acetone, acetonitrile and methanol, 9 min with ethanol, to a maximum of 20 min with isopropanol and binary mixtures with higher percentages of water. Under our operating conditions, the reproducibility of the R_F values was comparable to that obtained normally on silica gel plates, when particular attention is not paid to the ambient moisture (the relative standard deviation of the R_F values is ± 0.04). The detection limit for the barbiturates was at least an half that previously found on RP-18 plates[2] and in parallel experiments performed on silica gel plates. As regards the steroids, 0.2–0.3 μg of these compounds can be detected.

CONCLUSION

The amino-bonded silica gel pre-coated plates may be employed with all the twelve pure organic solvents tested and with binary mixtures with very high water contents. The development time is always very short, normally from 4–5 min to a maximum of 20 min with isopropanol.

The retention of barbiturates and steroids generally differs significantly from that found on silica gel plates. Very good selectivity was found for the steroids. It seemed that the retention order was related to the possibility of hydrogen bonding between the amino groups of the packing and the phenolic A ring and/or the alcoholic OH groups of the steroids. The barbiturates, which are weak diprotic acids, in contrast were all strongly retained, whereas the retention sequence seemed to depend on the substituents in position 5; generally, the barbiturates with aromatic side-chains were more retained than those with aliphatic side-chains.

The relative solvent strength on NH_2-modified plates did not follow an order resembling that reported for other polar supports, such as alumina. The barbiturates

were less retained with tetrahydrofuran or acetone than with more polar solvents, such as nitromethane or isopropanol. The subdivision of the solvents into several classes, as postulated for silica supports[15,16], may explain some of the differences in the chromatographic behaviour of the compounds examined, differences related to the eluent and to the compound employed. These results may be very useful when choosing the eluent for a given separation.

The retention sequence of the barbiturates and steroids on NH_2-modified plates remained almost the same for all of the eluents tested; on this type of support the variation of the mobile phase composition seemed generally to have less influence on increasing the separation selectivity than occurs with alumina or silica.

The HPTLC data agree well with HPLC results[11,12], and the data may also be useful in column chromatography.

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