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HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY OF INDOLE DERIVATIVES ON LAYERS OF SIL C_{18}-50 UNTREATED OR IMPREGNATED WITH N-DODECYLPYRIDINIUM CHLORIDE AND ON AMMONIUM TUNGSTOPHOSPHATE

LUCIANO LEPRI*, PIER GIORGIO DESIDERI and DANIELA HEIMLER
Institute of Analytical Chemistry, University of Florence, Via Gino Capponi 9, 20121 Florence (Italy)
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SUMMARY

The effects of indole structure and eluent pH and composition on the retention of 22 indole derivatives on layers of Sil C_{18}-50 untreated or impregnated with 4% N-dodecylpyridinium chloride were evaluated. Indole derivatives with a carboxyl group in the molecule are highly retained in acidic solution and have minimum retention at strongly alkaline pH.

The optimum coinditions for the separation of the greatest number of compounds were investigated and theoretical and experimental two-dimensional chromatograms (which allow the separation of 20 compounds) are reported.

On home-made layers of ammonium tungstophosphate (AWP), solutions of ammonium nitrate at different concentrations were employed for the separation of those indole derivatives which exhibit similar characteristics on Sil C_{18}-50 plates. The AWP layers allow the determination of 5-hydroxyindole-3-acetic acid in urine at 2 mg per 24 h levels.

INTRODUCTION

Chromatographic techniques have been extensively employed in the analysis of indole derivatives, most of which play an important role both in clinical chemistry and in plant physiology[1–4]. In the thin-layer chromatography (TLC) of these compounds, hydrophilic stationary phases such as silica gel and cellulose have traditionally been used[5–12]; in column chromatography, in contrast, non-polar stationary phases of silanized silica gel have been employed[13–15].

As in previous studies we achieved satisfactory results in the separation of organic compounds with acidic or basic characteristics on layers of silanized silica gel, untreated or impregnated with detergents[16,17], and also on ammonium tungstophosphate (AWP)[17,18], we thought it would be of interest to employ reversed-phase high-performance TLC (HPTLC) in the study of the retention and separation of indole derivatives. In contrast to column chromatography, the two-dimensional technique can be used, eluting in two directions with non-aqueous and aqueous mo-

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TABLE I

R_F VALUES OF INDOLE DERIVATIVES ON PLATES OF SIL C_{18}-50 UNTREATED OR IMPREGNATED WITH 4% N-DPC

Eluents: (A) water–methanol–acetic acid (59:40:1); (B) 0.1 *M* ammonia in 40% methanol; (C) hexane–ethyl acetate–acetic acid (72:27:1); (D) 0.1 *M* ammonia + 0.1 *M* ammonium chloride in 40% methanol; (E) 0.5 *M* ammonia + 0.5 *M* ammonium chloride in 40% methanol; (F) hexane–ethyl acetate–acetic acid (67:32:1).

No.	*Compound*	R_F						*Amount* (*µg*)
		Sil C_{18}-50			*Sil C_{18}-50 + 4% N-DPC*			
		A	*B*	*C*	*D*	*E*	*F*	
1	Indole	0.16	0.13	0.97	0.07	0.07	0.82	0.2
2	Tryptamine	0.47	0.02	0.00	0.22	0.22	0.02	0.2
3	Tryptophan	0.64	0.67	0.00	0.32	0.39	0.00	0.2
4	Serotonin	0.62	0.08	0.00	0.42	0.43	0.00	0.2
5	Indole-2-carboxylic acid	0.34	0.78	0.63	0.05	0.11	0.11	0.5
6	Indole-5-carboxylic acid	0.69	0.89	0.75	0.17	0.33	0.37	0.2
7	Indole-3-acetic acid	0.62	0.84	0.61	0.10	0.21	0.13	0.2
8	5-Hydroxyindole-3-acetic acid	0.78	0.92	0.18	0.22	0.40	0.02	0.2
9	Indole-3-propionic acid	0.22	0.77	0.75	0.07	0.15	0.24	0.2
10	Indole-3-butyric acid	0.14	0.69	0.81	0.05	0.10	0.36	0.2
11	Indole-3-glyoxylic acid	0.61	0.82	0.00	0.10	0.21	0.00	2.0
12	Indole-3-lactic acid	0.59	0.80	0.04	0.10	0.23	0.00	0.5
13	Indole-3-acrylic acid	0.15	0.74	0.64	0.06	0.13	0.22	1.0
14	Indole-3-acetamide	0.41	0.36	0.34	0.25	0.25	0.16	0.3
15	Indole-3-glyoxylamide	0.25	0.20	0.52	0.14	0.13	0.22	4.0
16	Indole-3-ethyl acetate	0.09	0.07	0.98	0.04	0.04	0.75	0.2
17	Indole-3-aldehyde	0.25	0.18	0.60	0.12	0.12	0.30	4.0
18	Indole-3-acetaldehyde	0.32	0.22	0.00* 0.96**	0.11	0.10	0.00* e.s.**	1.0
19	Indole-3-ethanol	0.29	0.23	0.74	0.14	0.14	0.42	0.3
20	Indole-3-acetone	0.24	0.18	0.95	0.11	0.12	0.70	0.5
21	Indole-3-acetonitrile	0.23	0.17	0.95	0.09	0.09	0.68	0.2
22	Isatin	0.42	0.40	0.78	0.26	e.s.	0.50	1.0

bile phases. Non-aqueous eluents have never been employed in the column chromatography of indole derivatives.

EXPERIMENTAL

Standard solutions were prepared by dissolving indole derivatives (Sigma, St. Louis, MO, U.S.A.) in water–methanol (1:1).

The sample volume was 0.2–0.5 μl of the desired indole solution for Sil C_{18}-50 plates (Macherey, Nagel & Co., Düren, G.F.R.), untreated or impregnated with 4% N-dodecylpyridinium chloride (Merck, Darmstadt, G.F.R.) and 0.5–1 μl for the AWP layers. With indole-3-glyoxylic acid, 3-glyoxylamide and -3-aldehyde, samples of 2 μl were deposited on both layers. The amounts used on Sil-C_{18}-50 plates are reported in Table I.

Detection was performed by spraying with 1% *p*-dimethylaminobenzaldehyde solution in concentrated hydrochloric acid–methanol (1:1) and heating the layers at 50°C for 20 min.

The impregnation of the Sil C_{18}-50 plates with N-dodecylpyridinium chloride (N-DPC) and the preparation of AWP and its layers were carried out as described previously[17,19,20]. The migration distance was 6 cm for Sil C_{18}-50 plates and 10 cm for AWP, unless stated otherwise. All the measurements were carried out at 25°C using a Desaga thermostatic chamber.

RESULTS AND DISCUSSION

Sil C_{18}-50 plates

Sil C_{18}-50 plates were used, because with the compounds concerned RP-18 plates generally give elongated spots and eluents containing at least 60% of methanol must be used in order to achieve reasonable elution times. Such high percentages of methanol give both a smaller retention and an unsatisfactory resolution. On Sil C_{18}-50 plates, in contrast, compact spots are obtained and eluents with a methanol content of 40% can be used as the elution time is about 50 min for a migration distance of 6 cm.

Table I lists on the chromatographic characteristics of indole and 21 of its derivatives on Sil C_{18}-50 plates as a function of composition and pH of the eluent.

Elution with water–methanol–acetic acid (59:40:1) (A) gives significant differences in the retentions of compounds, which may be compared with those obtained on LiChrosorb RP-18 columns with ethanol–1% acetic acid (20:80)[13]. The most interesting separations concern: (a) members of one homologous series (*i.e.*, indole-3-acetic, -3-propionic and -3-butyric acids, whose retention increases with increase in the number of the CH_2 groups in the side-chain); (b) some derivatives of indole-3-acetic acid with different functional groups (*i.e.*, 5-hydroxyindole-3-acetic acid, indole-3-acetamide, indole-3-acetaldehyde, indole-3-acetonitrile and indole-3-ethyl acetate) and indole-3-glyoxylic acid from its amide; it should be noted that indole-3-acetamide, in contrast to the results obtained in column chromatography, is more strongly retained than indole-3-acetic acid owing to its higher hydrophobicity, and that indole-3-acetaldehyde can be separated from indole-3-acetic acid; (c) pairs of isomers such as indole-2-carboxylic and -5-carboxylic acids; the higher retention of the first compound must be ascribed to its lower polarity[13].

With more acidic eluents (such as water–methanol–acetic acid, 54.3:40:5.7) no significant differences are observed in the chromatographic behaviour of most compounds, except indole-3-glyoxylic acid (R_F = 0.65) and indole-3-lactic acid (R_F = 0.51), which are well separated. In contrast, an increase in the apparent pH of the eluent results in a smaller retention of the indole derivatives with a carboxyl group owing to the deprotonation of this group. Considerable differences in the chromatographic behaviour of such indole derivatives in comparison with an acidic medium are observed for elution with 0.1 *M* ammonia in 40% methanol (B in Table I). Under these elution conditions the compounds without a carboxylic group are more strongly retained than with acidic eluents. Serotonin and tryptamine remain practically at the point of application as they are in the non-ionic form, which is the most strongly adsorbed from the stationary phase.

The presence of an inorganic salt in the eluent (3% potassium chloride) does not result in large differences in the R_F values of the indole derivatives in acidic media, where most compounds are predominantly in the non-ionic form. In alkaline solution a considerable increase in the retention of compounds with a carboxyl group is observed. Such an occurrence has already been pointed out for phenols and for dinitrophenylamino acids on layers of silanized silica gel alone or impregnated with anionic detergents[18,21].

With non-aqueous eluents, such as hexane–ethyl acetate–acetic acid (C in Table I), the elution sequence of the indole derivatives is completely different from that found with aqueous solutions. Further, the elution time decreases to 15 min. The presence of acetic acid in the eluent, even at low percentages, accounts for the compactness of the spots. It is significant that the composition of this eluent is very close to that which gave the best results in the separation of dinitrophenylamino acids on RP-18 plates[21].

As shown by the data in Table I (C), compounds with marked polar characteristics, such as tryptamine, tryptophan, serotonin, indole-3-glyoxylic acid and indole-3-lactic acid, remain at the point of application, whereas those with hydrophobic characteristics (indole, indole-3-acetone, indole-3-acetaldehyde and indole-3-ethyl acetate) migrate with the solvent front. Such behaviour agrees with the assumption of an adsorption mechanism on a polar stationary phase[21].

Sil C_{18}-50 plates impregnated with 4% N-DPC

Layers impregnated with anionic detergents, such as dodecylbenzenesulphonic acid, cannot be used as most indole derivatives yield several spots owing to a decomposition process promoted by the strong acidity of such detergents. Some compounds, after their deposition on the layer and evaporation of the solvent, give rise to coloured spots. On layers of Sil C_{18}-50 impregnated with 4% N-DPC, in contrast, such disadvantages are not observed when eluting with aqueous–organic solutions containing ammonia buffer at different concentrations and with mixtures of non-aqueous solvents.

Elution with 0.1 *M* ammonia–0.1 *M* ammonium chloride in 40% methanol (D in Table I) results in a stronger retention of the indole derivatives than that obtained on untreated plates when eluting with water–methanol–acetic acid (A). Such behaviour can be ascribed to an anion-exchange process and/or to a higher hydrophobicity of the stationary phase owing to the presence of the detergent. On this layer some

urinary indoles, such as tryptophan, tryptamine, serotonin and indole-3-acetic acid, can be separated. With the above-mentioned eluent, tryptamine and serotonin exhibit a lower retention than on untreated layers eluted with 0.1 *M* ammonia in 40% methanol, as such compounds are mainly in the charged protonated form which gives rise to repulsive forces with the functional group of the detergent adsorbed on the stationary phase.

An increase in the ammonia and ammonium chloride concentrations in the eluent (E in Table I) results in an increase in R_F for those indoles which contain a carboxyl group, and therefore a negative charge in the molecule. Plotting the R_M values as a function of log [NH_4Cl] in the 0.1–1 *M* concentration range, straight lines are obtained with slopes of 0.25 for tryptophan and about 0.5 for the other indoles containing a carboxyl group. Such values indicate the occurrence of an anion-exchange process between these compounds and the detergent sorbed on the layer.

Elution with non-aqueous solvents (F in Table I) allows a sharp separation among indole-3-acetic, -propionic and -butyric acids and a better resolution of the neutral indole derivatives, which run with the solvent front in the absence of the detergent.

Two-dimensional chromatograms

As the separation of all or most of the indole derivatives cannot be achieved with only one elution, we used the two-dimensional technique on untreated Sil C_{18}-50 plates. Such plates are suitable for this technique, whereas impregnated layers are less advantageous as the elution in the first direction changes the characteristics of the stationary phase.

The data in Table I indicate that the optimum conditions for the separation of the largest number of indoles are elution in the first direction with hexane–ethyl acetate–acetic acid (72:27:1) and in the second direction with 0.1 *M* ammonia in 40% methanol.

Fig. 1a shows the theoretical chromatogram of 21 compounds on the basis of their R_F values and Fig. 1b the experimental chromatogram. After the first elution the plate was dried at room temperature for 1 h in order to allow the complete evaporation of the solvents. With the two-dimensional technique 20 indole derivatives were separated. The spots were identified from their positions on the chromatogram and also from their colours.

Layers of ammonium tungstophosphate (AWP)

We used layers of AWP + $CaSO_4 \cdot \frac{1}{2}H_2O$ (4:2), because with this ratio compact spots and significant differences in the retentions of compounds were obtained. Fig. 2 shows the chromatographic characteristics of indole derivatives obtained on eluting with 1 *M* ammonium nitrate and some separations which are important from an analytical point of view, such as that between indole-3-acetone and indole-3-acetonitrile, which exhibit the same chromatographic behaviour on Sil C_{18}-50 plates untreated or impregnated with 4% N-DPC. The characterization of the above-mentioned indoles in the mixture is also supported by the different colours of their spots.

Another important separation of the five urinary indoles may be achieved with only one elution (see Fig. 2), while on Sil C_{18}-50 plates a two-dimensional development must be effected. An increase is the concentration of ammonium nitrate in the

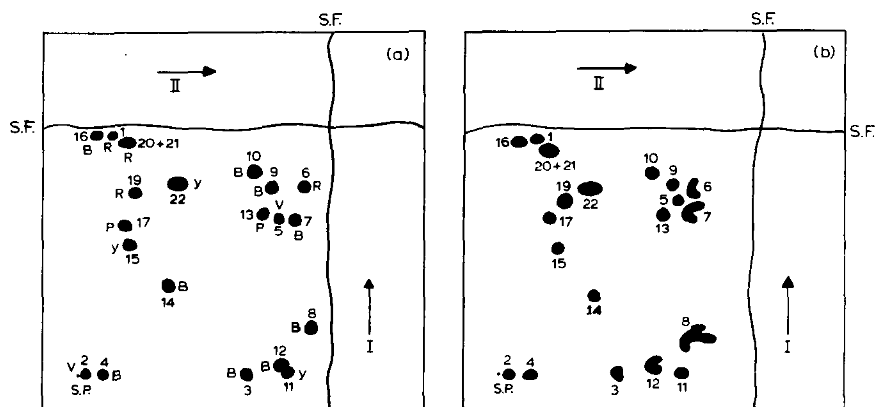

Fig. 1. (a) Theoretical and (b) experimental two-dimensional chromatograms on Sil C_{18}-50 plates. Eluents: in the first direction, hexane–ethyl acetate–acetic acid (72:27:1); in the second direction, 0.1 M ammonia in 40% methanol. Indole derivatives as in Table I. Colours of the spots: B = blue; Y = yellow; V = violet; R = red; P = pink. S.P. = start point; S.F. = solvent front.

eluent (3 M) results in larger differences among the R_F values only for the three indole derivatives that contain charged protonated amine groups which share in a cation-exchange process. Sharp differences in the R_F values are shown by indole-3-glyoxylic acid (R_F = 0.49) and indole-3-lactic acid (R_F = 0.33), which may be separated with this eluent.

Indole has not been detected on this layer in the amount normally used. Layers of AWP + $CaSO_4 \cdot \frac{1}{2}H_2O$ in a ratio of 4:2 are suitable for the determination of 5-hydroxyindole-3-acetic acid in urine (normal excretion range 2–8 mg per 24 h).

This tryptophan metabolite, if excreted in abnormally larger amounts (20–400

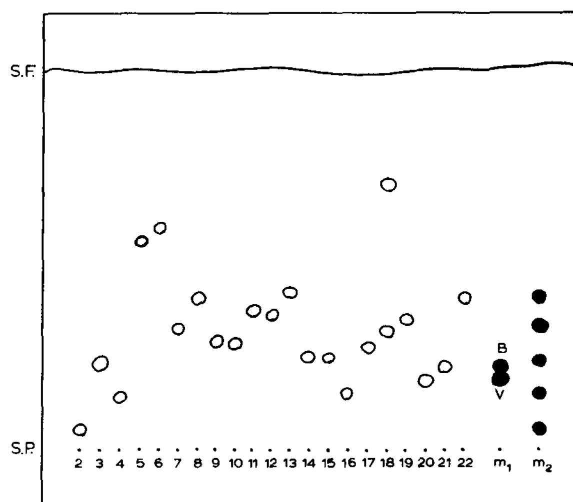

Fig. 2. Thin-layer chromatogram on AWP + $CaSO_4 \cdot \frac{1}{2}H_2O$ (4:2). Eluent: 1 M NH_4NO_3. Indole derivatives as in Table I. m_1 = mixture of 20 and 21; m_2 = mixture of 2, 3, 4, 7 and 8. Colours of the spots: B = blue; V = violet. S.P. = start point; S.F. = solvent front.

mg per 24 h), indicates the presence of carcinogens[1]. In order to extract 5-hydroxy-indole-3-acetic acid, 2.5 g of sodium chloride and 0.5 ml of concentrated hydrochloric acid are added to 10 ml of urine, then the sample is shaken twice for 5 min with 10 ml of diethyl ether. The extracts are combined and evaporated to dryness at room temperature under a flow of nitrogen and the residue is dissolved in 0.1 ml of methanol. Volumes of 0.5 and 2 μl of the methanol solution are deposited on the layer and eluted with 1 *M* ammonium nitrate. The amount excreted is evaluated by comparison with a standard run at the same time. Amounts of 5-hydroxyindole-3-acetic acid as low as 2 mg per 24 h can be detected.

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