THE ADAPTATION OF A MODIFIED ADAMKIEWICZ-HOPKINS TEST TO THE DETECTION OF INDOLE COMPOUNDS AND THE QUANTITATIVE DETERMINATION OF TRYPTOPHAN BY TLC

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DIAMANTSTEIN AND EHRHARDT[1] and STAHL AND KALDEWEY[2] have detected and identified indole compounds on plates covered with silica gel by using the fluorescence reaction described by PROCHÁZKA[3] and the Ehrlich reaction[4].

We became interested in yet another colour reaction, which is very sensitive and has been used for some time to detect the presence of indole compounds in solution, known as the Adamkiewicz-Hopkins test. This test was modified by FISCHL[5] for use in quantitative estimations of indole compounds in solution. When we used this modified test in our investigation of tryptophan in plasma and blood corpuscles[6], we did not obtain repeatable results.

After further modification of the test we were able to use it for quantitative determinations of indole compounds in biological material[7] and this reaction has now been adapted for use on plates covered with silica gel and powdered cellulose. This test could not be used for paper chromatography because of the destructive power of the concentrated acids in the reagent.

EXPERIMENTAL

Materials

The materials used and their sources are listed below:

(1) DL-Tryptophan (B.D.H.).
(2) Serotonin, creatine sulphate complex (Merck).
(3) DL-5-Hydroxytryptophan (Fluka A.G.).
(4) (Indole-3)-acetic acid (Merck).
(5) 5-Hydroxyindoleacetic acid (manufacturer unknown).
(6) (Indole-3)-propionic acid (B.D.H.).*

METHOD

In our investigations we employed thin-layer chromatography (TLC) with silica gel G 22 and powdered cellulose MN 300 as sorbents. The development was carried out on plates 20 × 20 cm covered with sorbent by means of the spreader made by Desaga. The thickness of the layer of sorbent was 0.3 mm. The separation was carried out in 10 cm sections.

* The numbering of the indole compounds corresponds to that given in Figs. 1–6.

The two solvents used for development were: isopropanol–ammonia 25 %–water (20:1:2) and *n*-butanol–glacial acetic acid–water (15:3:5). Identification was by either the fluorescence method in U.V. light, Ehrlich's reagent (10 % *p*-dimethylaminobenzaldehyde in concentrated HCl–acetone, 1:4), or the modified Adamkiewicz-Hopkins reagent (acetic acid containing 56 mg Fe/l, concentrated H_2SO_4).

The quantitative estimations were made by means of a Pulfrich photometer with an Elpho attachment, in containers 0.5 cm long and an S 53 filter.

RESULTS

The separation of indole compounds on plates covered with silica gel and cellulose was compared; the amount of standard substance deposited was 3 μg. The plates covered with cellulose were activated at 105° for 15 min, while the plates with silica gel were not activated but dried at 20° for 12 h. For results see Figs. 1 and 2.

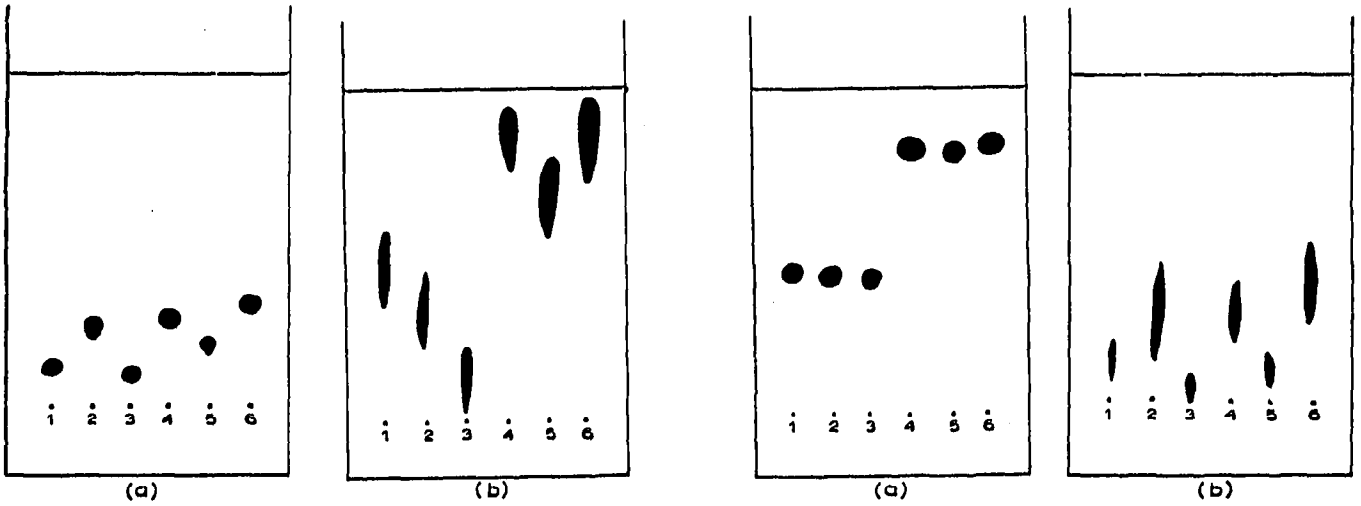

Fig. 1a. Thin-layer chromatogram of indole derivatives on silica gel G 22 with butanol–HAc–water (15:3:5) as solvent.

Fig. 1b. Thin-layer chromatogram of indole derivatives on cellulose MN 300 with butanol–HAc–water (15:3:5) as solvent.

Fig. 2a. Thin-layer chromatogram of indole derivatives on silica gel G 22 with isopropanol–ammonia 25 %–water (20:1:2) as solvent.

Fig. 2b. Thin-layer chromatogram of indole derivatives on cellulose MN 300 with isopropanol–ammonia 25 %–water (20:1:2) as solvent.

Some separations by a two-dimensional technique were also carried out. The following solvent systems were used:

(A) I — chloroform–glacial acetic acid (95:5)
II — methyl acetate–isopropanol–ammonia 25 % (45:35:20)

(B) I — methyl acetate–isopropanol–ammonia 25 % (45:35:20)
II — chloroform–methanol–glacial acetic acid (75:20:5)

(C) I* — isopropanol–ammonia 25 %–water (20:1:2)
II* — *n*-butanol–glacial acetic acid–water (15:3:5).

* The solvents used by us.

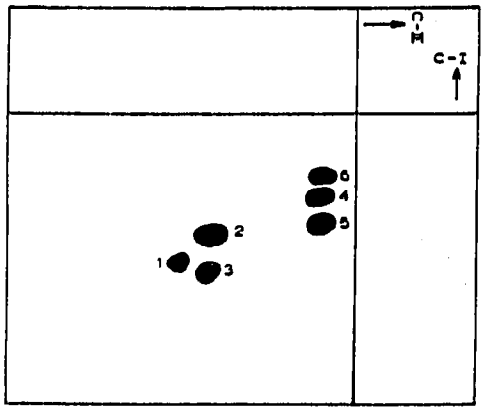

Fig. 3. Thin-layer two-dimensional chromatogram of indole derivatives mixture on silica gel G 22 with isopropanol–ammonia 25%–water (20:1:2) (I) and butanol–HAc–water (15:3:5) (II) as solvents.

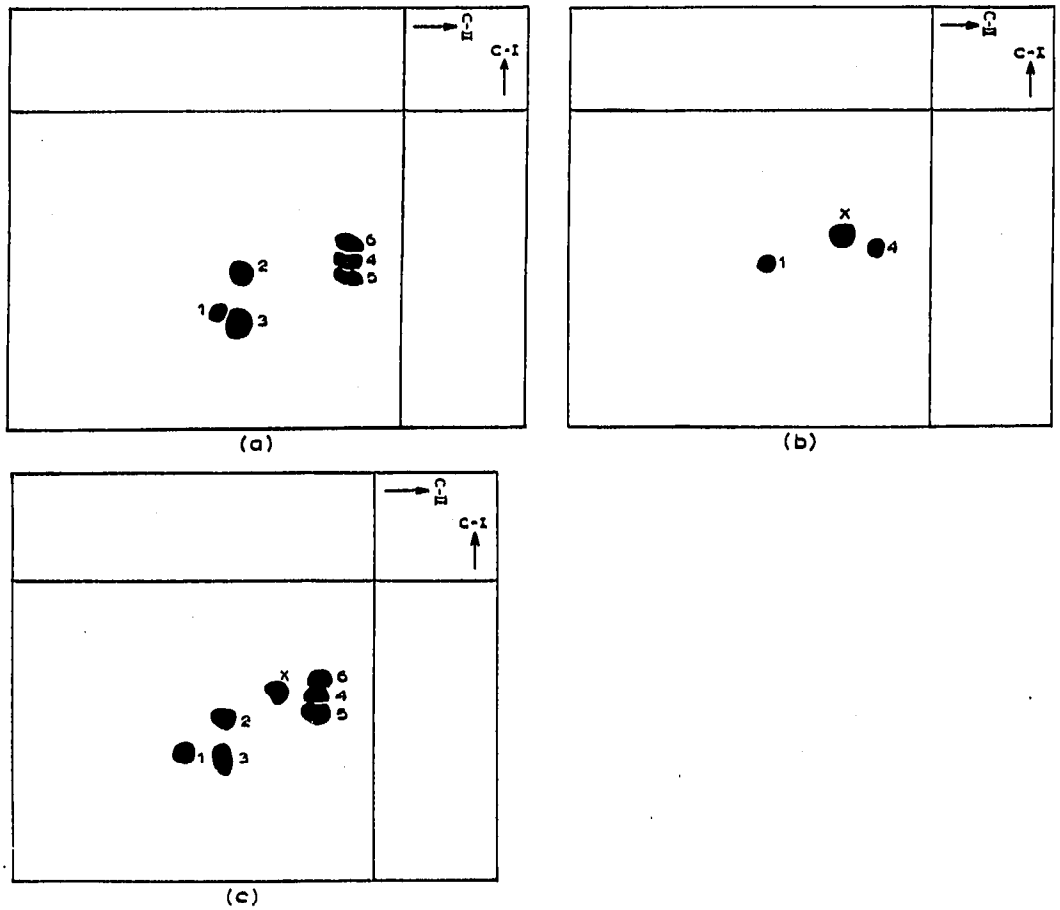

Fig. 4a. Thin-layer two-dimensional chromatogram of indole derivatives mixture on silica gel G 22 with isopropanol–ammonia 25%–water (20:1:2) (I) and butanol–HAc–water (15:3:5) (II) as solvents.

Fig. 4b. Thin-layer two-dimensional chromatogram of indole derivatives in urine on silica gel G 22 with isopropanol–ammonia 25%–water (20:1:2) (I) and butanol–HAc-water (15:3:5) (II) as solvents.

Fig. 4c. Thin-layer two-dimensional chromatogram of urine with added indole derivatives on silica gel G 22 using isopropanol–ammonia 25%–water (20:1:2) (I) and butanol–HAc–water (15:3:5) (II) as solvents.

TABLE I

COMPARISON OF THE SENSITIVITY OF INDOLE DERIVATIVES TO SPECIFIC COLOUR TESTS ON SILICA GEL AND CELLULOSE

Indole derivatives	*Silica gel G 22*				*Cellulose MN 300*			
	Ehrlich test		*Modified Adamkiewicz-Hopkins test*		*Ehrlich test*		*Modified Adamkiewicz-Hopkins test*	
	Sensitivity (μg)	*Colour*	*Sensitivity (μg)*	*Colour*	*Sensitivity (μg)*	*Colour*	*Sensitivity (μg)*	*Colour*
Tryptophan	0.03	pink-violet	0.05	yellow-violet	0.02	violet	0.03	violet
Serotonin-creatine sulphate	0.04	grey-blue	0.03	yellow-blue	0.01	violet-blue	0.03	violet
5-Hydroxy-tryptophan	0.03	violet-blue	0.03	yellow-blue	0.02	violet-blue	0.03	violet-blue
3-Indolylacetic acid	0.02	violet	0.01	violet	0.01	violet	0.01	violet
5-Hydroxy-indolyl-acetic acid	0.02	blue	0.03	blue	0.02	violet-grey	0.03	violet-grey
3-Indolylpropionic acid	0.03	violet	0.01	grey-violet	0.01	violet	0.03	violet-pink

The separation employing solvent system C is shown in Fig. 3.

The sensitivity of various indole derivatives, on silica gel and on cellulose, subjected to the Ehrlich test and to the Adamkiewicz-Hopkins test, is given in Table I.

Table II shows the R_F values of various indole derivatives separated on silica gel. A comparison is made between the separation obtained in isopropanol–ammonia 25 %–water (20:1:2) and *n*-butanol–acetic acid–water (15:3:5).

Two-dimensional chromatograms of indole derivatives on silica gel and of indole compounds in urine and plasma are shown in Figs. 4 and 5. The detection of indole compounds in the presence of amino acids is shown in Fig. 6.

TABLE II

R_F VALUES OF INDOLE DERIVATIVES ON SILICA GEL G 22

Indole derivatives	*Solvents*	
	Isopropanol–ammonia–water (20:1:2)	*n-Butanol–acetic acid–water (15:3:5)*
Tryptophan	0.11	0.43
Serotonin-creatine sulphate	0.23	0.42
Hydroxy-tryptophan	0.08	0.38
3-Indolylacetic acid	0.26	0.80
5-Hydroxyindolylacetic acid	0.18	0.78
3-Indolylpropionic acid	0.31	0.82

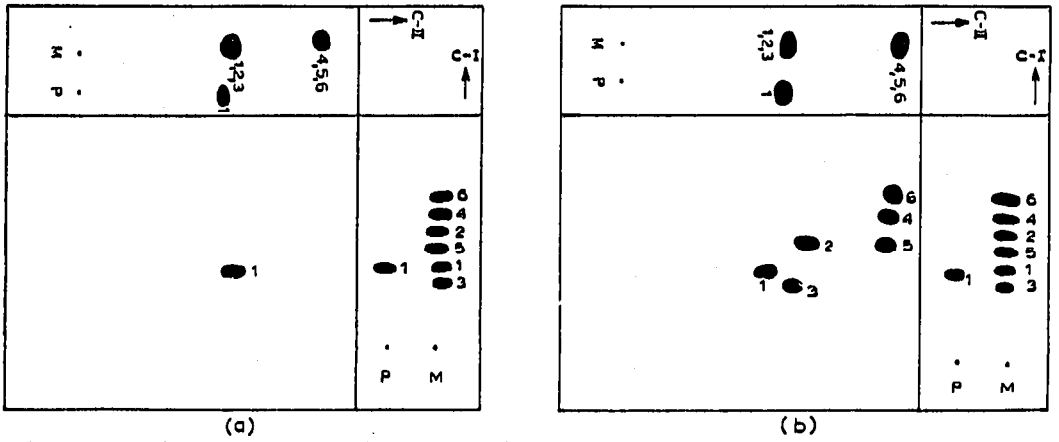

Fig. 5a. Thin-layer two-dimensional chromatogram of human plasma on silica gel G 22 with isopropanol–ammonia 25 %–water (20:1:2) (I) and butanol–HAc–water (15:3:5) (II) as solvents. P = plasma; M = indole derivatives mixture.

Fig. 5b. Thin-layer two-dimensional chromatogram of human plasma with added indole derivatives on silica gel G 22 using isopropanol–ammonia 25 %–water (20:1:2) (I) and butanol–HAc–water (15:3:5) (II) as solvents. P = plasma; M = indole derivatives mixture.

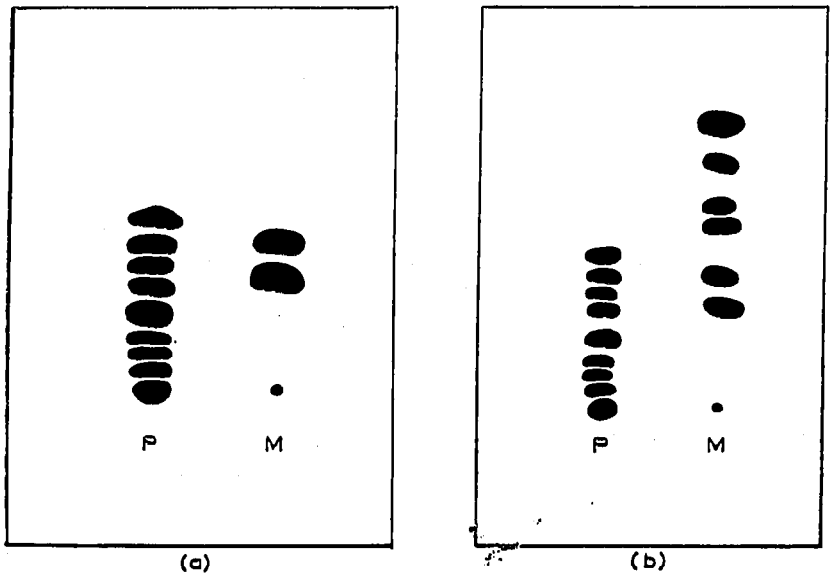

Fig. 6a. Thin-layer one-dimensional chromatogram of amino acids (P) and indole derivatives mixture (M) on silica gel G 22 with isopropanol–ammonia 25 %–water (20:1:2) as solvent. Ninhydrin detection only.

Fig. 6b. Thin-layer one-dimensional chromatogram of amino acids (P) and indole derivatives mixture (M) on silica gel G 22 with isopropanol–ammonia 25 %–water (20:1:2) as solvent. Ninhydrin and Ehrlich tests.

Quantitative estimation

After carrying out the separation of the mixture of indole compounds on plates covered with silica gel or cellulose, and identifying tryptophan by the fluorescence test, the gel corresponding to the tryptophan spot is scraped off the plate. It is eluted in 0.5 ml of water, 1.5 ml of glacial acetic acid containing 56 mg Fe/l and 1 ml of concentrated sulphuric acid. After mixing, the liquid is centrifuged and the tryptophan is estimated in the resulting supernatant. The extinction value for tryptophan

is calculated from the standard curve (see Fig. 7). The loss of tryptophan during elution and the development of the chromatograms was determined as shown in Table III.

TABLE III

RECOVERY OF TRYPTOPHAN AFTER TLC

Tryptophan (μg)	*Without development*		*n-Butanol–acetic acid–water (4:1:1)*			
			Silica gel G 22		*Cellulose MN 300*	
	(μg)	(%)	(μg)	(%)	(μg)	(%)
5	3.5– 4.5	70–90	2.5– 4.0	50–80	2.5– 4.0	50–80
10	6.5– 9.5	65–95	5.5– 9.0	55–80	8.5– 9.0	85–90
15	14.0–14.5	90–97	7.5–10.0	50–61	12.0–15.0	80–100
20	17.5–20.0	87–100	12.0–15.0	60–75	17.0–20.0	90–100
25	22.5–25.0	90–100	15.0–18.0	60–72	23.0–24.5	90–95
30	26.0–28.0	87–90	17.5–20.0	58–70	26.0–27.0	87–90
40	30.0–38.0	80–90	25.0–28.5	62–71	33.0–35.0	82–88

DISCUSSION

The separation of the indole compounds was carried out by thin-layer chromatography using silica gel (Merck) and powdered cellulose without plaster of Paris (Macherey-Nagel MN 300) as sorbents. A better separations was obtained on silica gel; the spots of the indole compounds are compact, clear and well-defined, while on cellulose they run into one another and are indistinct.

In the development of two-dimensional chromatograms we used the solvent systems A, B and C*. System C as used by STOWE AND THIMANN[8] was found to be the best for the substances under investigation. For one-dimensional chromatography the best separation was obtained using solution C I.

Comparing the colour tests of Ehrlich and Adamkiewicz-Hopkins, we found slight differences in their sensitivity. The deviations were in the range of 0.01–0.03. The modified Adamkiewicz-Hopkins test is more suitable for the identification of indole compounds, because the colour remains longer in comparison with the Ehrlich test. When the Ehrlich test is used for the detection of indole compounds in urine, urea gives a diffuse yellow spot; the Adamkiewicz-Hopkins test gives no colouring with urea. The sensitivities of these tests are similar, but that of the fluorescence tests is ten times greater.

Good results in the separation of indole compounds in urine are only obtained in 30 μl without desalting and in 20 μl of deproteinized plasma.

Development of the chromatograms with ninhydrin followed by the Ehrlich test enables the identification of indole compounds in the presence of amino acids to be carried out.

In the quantitative estimations of tryptophan from the plates without development the losses were within the range of 10–30 μg. During the development of the chromatograms in the butanol system, the losses of tryptophan from the gel plates

* The solvents used by us.

were about 40 %, while on cellulose they are not more than 20 %. Probably the greater losses on the gel are caused by the adsorption of the tryptophan on the plaster of Paris, which is a constituent of the gel.

From this investigation it can be seen that the Adamkiewicz-Hopkins test is equally good for the purposes of identification and quantitative estimation.

Attempts at quantitative estimations of other indole compounds are in progress.

SUMMARY

Indole compounds were separated by TLC on silica gel and cellulose and identified by the colour tests of Ehrlich and Adamkiewicz-Hopkins. The tests were carried out on standard substances and on biological materials containing indole compounds.

The modified Adamkiewicz-Hopkins test was adapted for the identification and quantitative estimation of indole compounds.

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