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A STUDY OF THE CHROMATOGRAPHIC BEHAVIOUR OF TRYPTOPHAN METABOLITES AND RELATED COMPOUNDS BY CHROMATOGRAPHY ON THIN LAYERS OF SILICA GEL

PART I. QUALITATIVE SEPARATION

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SUMMARY

Using a two-dimensional thin-layer chromatographic procedure with thin layers of Silica Gel GF_{254}, thirty-two tryptophan metabolites and related compounds have been unambiguously separated on a single chromatogram. The separation takes only about 3 h and can be applied to the identification of these compounds in urine.

INTRODUCTION

There are several different pathways available for the metabolism of tryptophan in the human[1]. Accordingly, it is not surprising that many clinical conditions have been reported in which disturbed tryptophan metabolism is stated to be involved[2,3]. Another effect of the complex metabolism of tryptophan is that the metabolites which have so far been identified in normal and pathological human urine differ widely in their chemical structure and, therefore, their chromatographic properties. As a consequence, the separation of tryptophan metabolites is not easily carried out using simple chromatographic techniques. Over the past few years much work has been performed on the application of thin-layer chromatographic (TLC) procedures to this problem[4-6]. However, great difficulty has been experienced, to date, in separating more than about ten tryptophan metabolites on the one two-dimensional thin layer[5]. Prior to a study of the occurrence of tryptophan metabolites in the urine of normal and mentally retarded children, we decided to re-investigate the TLC properties of the compounds.

The present paper describes the separation achieved by a new two-dimensional solvent system on thin layers of silica gel. The unambiguous resolution of a mixture of thirty-two of the more common tryptophan metabolites and related compounds is described and the chromatographic properties of a total of forty compounds are

listed. The new solvent system is then applied to the identification of tryptophan metabolites in normal and pathological samples of urine.

EXPERIMENTAL

Apparatus

The thin-layer equipment used throughout this work was supplied by Shandon*. Solutions were applied to the thin layers by means of capillary pipettes (Microcaps)** of 1 μl capacity. The UV equipment used for viewing the chromatograms at wavelengths of 254 and 350 nm was a Camag Universal UV lamp and supplied by Camlab***.

Materials and methods

Adsorbent. At present, paper chromatographic (PC) procedures are widely and successfully used for the separation of the tryptophan metabolites[7], but TLC offers many advantages with regard to speed and sensitivity. As a consequence, thin layers of cellulose (MN-300) (Camlab) were prepared[8] since such systems are reported to correspond well with PC systems[9]. However, when solvents previously employed for PC separations of the tryptophan metabolites were used[7], on the thin layers of cellulose highly distorted chromatograms were obtained. Preliminary work was therefore carried out in an attempt to find a suitable support[10]. From the results of these experiments Silica Gel GF_{254} (Merck)§ was chosen to be used throughout this work.

Solvents for chromatographic development§§. The solvents used throughout this work were of AnalaR or MFC grade with the exception of chloroform (GPR).

Detection reagent. The reagent used for the detection of indoles and related compounds was Ehrlich reagent. It consists of a solution of *p*-dimethylaminobenzaldehyde in concentrated hydrochloric acid (10% w/v). This was added to four times its volume of propanone. The reagent is unstable and was prepared just prior to use.

Urine samples. Several normal and pathological urine samples were obtained from children of both sexes after a period of overnight fasting. The samples were concentrated by the method of DALGLIESH[11] as soon as possible after arrival in the laboratory.

Preparation of thin layers. Five glass plates (20 × 20 cm) were first cleaned entirely free from grease by immersing them overnight in Haemosol§§§ solution and rinsing well with distilled water afterwards. A slurry of Silica Gel GF_{254} (30 g) and distilled water (60 ml) was prepared by homogenising the mixture for 30 sec using a high-speed electric propellor stirrer. Dry plates were then coated with the slurry, using Shandon equipment, to provide a layer initially 300 μ thick. When the surface of the layer became matt, the plates were placed in a rack and stored horizontally

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* Shandon Scientific Co., 65 Pound Lane, London NW10, Great Britain.
** Drummond Scientific Co., Philadelphia, Pa., U.S.A.
*** Camlab (Glass Ltd.)., Cambridge, Great Britain.
§ Agents Andermann and Co. Ltd., Tooley St., London, S.E.1.
§§ Hopkin and Williams Ltd., Chadwell Heath, Essex, Great Britain.
§§§ A. Cox (Surgical) Ltd., Edward Road, Coulsdon, Surrey, Great Britain.

overnight. Immediately prior to use the plates were placed in the oven and heated at 110° for 30 min.

Preparation of standard solutions. Stock solutions (1%, w/v) of the tryptophan metabolites and related compounds were prepared in propanone–water (1:1). When necessary the minimum amount of 1 *N* hydrochloric acid or 2 *N* sodium hydroxide was added to dissolve those compounds insoluble in propanone–water.

Application of solutions to thin layers. Spots were placed at a point 1.5 cm up from the lower edge of the layer and 1.5 cm inside from the left hand edge of the layer. The solvent front in each dimension (13 cm from the origin) was marked at the edges of the layer.

Solvents for chromatographic development. After much experimentation, two solvents were finally chosen which were theoretically capable of giving an excellent two-dimensional separation of tryptophan metabolites and related compounds[10]. These were: solvent A, which consisted of propanone–2-propanol–water–ammonia (0.88) (50:40:7:3), and solvent B, which consisted of chloroform–acetic acid–methanol–water (65:20:10:5).

Determination of optimum conditions for development. In order to obtain reproducible R_F data a study was made of some of the various parameters which are known to affect R_F values[12].

The effect of solvent equilibration time in the development tank on the reproducibility of R_F values was noted using the following procedure. A Shandon development tank was lined with Whatman No. 3MM chromatography paper and 100 ml of freshly prepared developing solvent were added. The paper was soaked by tilting the tank, and then pressed to the tank wall. The atmosphere in the tank was allowed to become saturated with solvent vapour for varying periods of time (30, 45 and 60 min) and overnight (18 h). In addition, the method due to SANKOFF AND SOURKES[13] was tried. In this procedure (S.S.) solvent from the previous day's chromatography was allowed to remain in the tank. Prior to development, the old solvent was discarded and replaced by 100 ml of fresh solvent. The tanks were again allowed to equilibrate for 30, 45 and 60 min. When ready, plates containing a selection (see Fig. 1) of fifteen of the test substances spotted along a line 1.5 cm up from the lower edge of the layer were placed into the development tank. When the solvent front had travelled 13 cm from the origin, the plates were removed from the tank and dried in a forced draught oven at 60° for 15 min. The plate was viewed under UV light at wavelengths 254 and 350 nm and the spots were noted. The plate was then sprayed with Ehrlich reagent and allowed to stand overnight at room temperature (22°). The positions of the spots were noted as they appeared and their R_F values determined. Five replicates for each solvent system were carried out on separate days.

A study was made of the effect of temperature and time of development upon both reproducibility of R_F data and the stability of the compounds. Thin layers were spotted as described above with a selection of fifteen of the compounds. The whole of each layer was sprayed with 15 ml of developing Solvent A and then immediately placed into a forced draught oven. They were then heated at differing temperatures (40°, 60°, 80° and 100°) for varying periods of time (15, 30 and 45 min). It has been shown (this paper) that the optimum conditions for tank saturation for Solvent B can be obtained by employing the method of SANKOFF AND SOURKES[13] and allowing

the fresh solvent to stand for 30 min. When cool, the plates were therefore developed in Solvent B under these conditions. The spots were revealed with Ehrlich reagent, and their positions, and whether or not decomposition had occurred (multiplicity of spots or reduced intensity of colour), were noted. The procedure was repeated, spraying with Solvent B, heating and then developing in Solvent A.

RESULTS AND DISCUSSION

In Fig. 1 are plotted the mean hR_F values of fifteen compounds in Solvent A under different conditions of tank saturation and different equilibration times. A study shows that the system has reached equilibrium at 30 min and that consequently

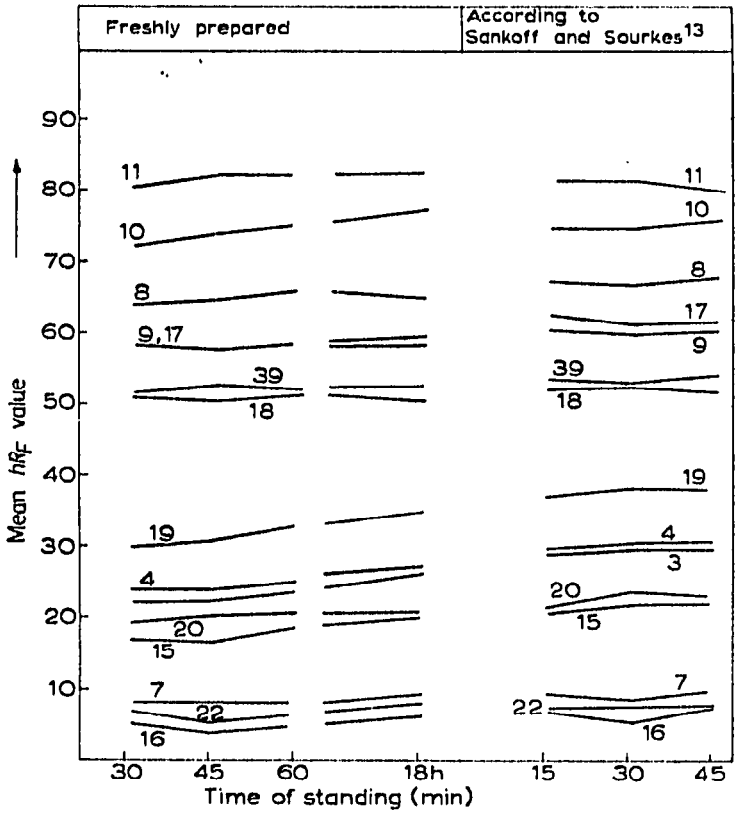

Fig. 1. Mean hR_F values of tryptophan metabolites and related compounds in the solvent system propanone–2-propanol–water–ammonia (0.88) (50:40:7:3) at different conditions of tank saturation. For key see Table IV.

hR_F values are little affected by further standing. Table I gives the mean standard deviation (S.D.) of the hR_F values of the fifteen compounds under the different conditions of equilibrium employed. From this it can be seen that the lowest S.D. (and hence the most consistently reproducible hR_F values) is obtained using the method of SANKOFF AND SOURKES[13] (S.S.) and allowing the fresh solvent to stand for 30 min before introducing the thin layer into the tank. Similarly, examination of Fig. 2 shows that equilibrium is not achieved using freshly prepared tanks. A study of Table I and Fig. 2 shows that the optimum conditions for chromatography in

TABLE I

MEAN STANDARD DEVIATION OF THE hR_F VALUES OF FIFTEEN TRYPTOPHAN METABOLITES IN SOLVENTS A AND B UNDER DIFFERENT CONDITIONS OF TANK SATURATION

For list of compounds used see Table IV.

Solvent	*Time of standing*						
	Freshly prepared				*According to* SANKOFF AND SOURKES[13]		
	30 min	*45 min*	*60 min*	*18 h*	*15 min*	*30 min*	*45 min*
A	1.55	1.58	1.78	2.68	1.22	1.04	1.28
B	1.30	1.79	2.00	2.24	1.00	1.18	1.08

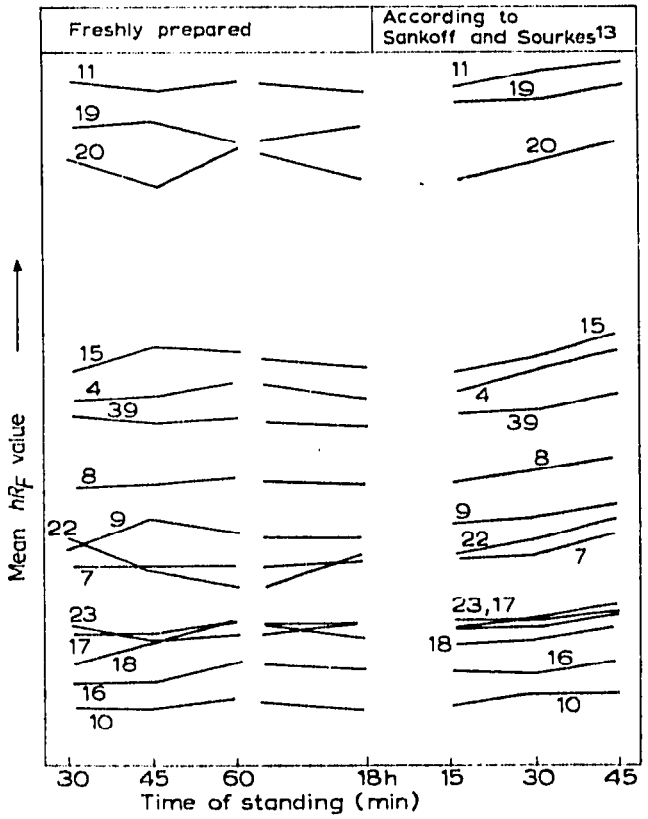

Fig. 2. Mean hR_F values of tryptophan metabolites and related compounds in the solvent system chloroform–acetic acid–methanol–water (65:20:10:5) at different conditions of tank saturation. For key see Table IV.

Solvent B are the same, that is, S.S. followed by allowing the fresh solvent to stand in the development tank for 30 min.

One-dimensional chromatography of the compounds in Solvents A and B gave R_F values which theoretically produced an excellent two-dimensional separation. Accordingly, it was necessary to ensure that the conditions for development in the second dimension were as near as possible to those obtained when the solvent was used for one-dimensional chromatography. Complete removal of the first-dimension

solvent is necessary because residual solvent is known to affect the chromatographic properties of a compound in the second dimension[14]. However, if too drastic conditions of removal are employed then breakdown of the compounds of interest may occur. It has been reported[15] for PC studies that solvent systems containing ammonia cause partial destruction of some tryptophan metabolites. Accordingly Solvent B, containing acetic acid, was used initially for development in the first dimension.

In Table II is given the absolute mean ΔhR_F values of the compounds in Solvent B after the plate had been treated with Solvent B. A study of this table shows that heating the plate at 80° for 45 min gave hR_F values which most nearly approached the optimum for Solvent A. Further, from Table II the standard deviation of the hR_F values at these conditions gives a value of 1.13, which shows good reproducibility. However, streaking of compounds with low hR_F values occurred,

TABLE II

DETERMINATION OF OPTIMUM CONDITIONS FOR REMOVAL OF SOLVENT B FROM THE THIN LAYER PRIOR TO CHROMATOGRAPHY OF TRYPTOPHAN METABOLITES IN SOLVENT A

Temperature (°C)	*ΔhR_F value*			*Standard deviation*		
	15 min	*30 min*	*45 min*	*15 min*	*30 min*	*45 min*
40	12.7	11.7	9.8	1.50	2.55	1.43
60	11.7	11.4	6.5	3.54	3.07	2.38
80	8.2	7.3	3.7	1.93	1.97	1.13
100	4.2	6.0	6.9	2.27	1.80	0.92

and when the plates were sprayed with Ehrlich reagent much fainter spots were obtained than with those heated at lower temperatures and shorter times. These effects indicate that breakdown of the compounds occurs under the conditions of removal of Solvent B at 80° for 45 min. Accordingly, it was necessary to try the effect of using the ammonia-containing solvent in the first dimension. From a study of Table III it can be seen that heating the plate at 60° to remove Solvent A gave low ΔhR_F values, indicating near optimum conditions for development in the second dimension using Solvent B. At this temperature the standard deviation for hR_F values was lowest when heated for 30 min and no decomposition of the compounds was noted under these conditions.

TABLE III

DETERMINATION OF OPTIMUM CONDITIONS FOR REMOVAL OF SOLVENT A FROM THE THIN LAYER PRIOR TO CHROMATOGRAPHY OF TRYPTOPHAN METABOLITES IN SOLVENT B

Temperature (°C)	*ΔhR_F value*			*Standard deviation*		
	15 min	*30 min*	*45 min*	*15 min*	*30 min*	*45 min*
40	5.8	7.5	5.5	0.90	0.83	0.91
60	1.9	1.3	1.9	0.92	0.70	1.28
80	4.3	5.6	5.5	0.84	1.53	0.53
100	5.1	4.7	5.3	0.84	0.59	1.09

The above results and discussion lead to the conclusion that the following conditions are necessary for optimum separation of the tryptophan metabolites. Solvent A is used for first-dimension development in a tank saturated using S.S. plus 30-min equilibration with the fresh solvent. When development is complete the solvent is removed by heating at 60° for 30 min. Cool plates are then developed in Solvent B in a tank saturated as described for Solvent A. When complete the excess solvent is removed by heating the plate at 60° for 5 min.

TABLE IV

hR_F VALUES OF TRYPTOPHAN METABOLITES ON THIN LAYERS OF SILICA GEL

Solvents: 1st dimension—Solvent A (propanone–2-propanol–water–ammonia (0.88), 50:40:7:3); 2nd dimension –Solvent B (chloroform–acetic acid–methanol–water, 65:20:10:5).

No.	*Compound*	*hR_F value*		*No.*	*Compound*	*hR_F value*	
		Solvent A	*Solvent B*			*Solvent A*	*Solvent B*
1	Indole	84	98	21	3-Hydroxyanthranilic acid	Decomposes	
2	3-Methylindole	85	99	22	Kynurenine	8	23
3	Indole-3-acetic acid	18	85	23	3-Hydroxykynurenine	9	17
4	Indole-3-lactic acid	24	40	24	*o*-Aminophenol	79	45
5	Indole-3-pyruvic acid	Decomposes		25	*p*-Aminohippuric acid	21	33
6	Indole-3-acrylic acid	25	85	26	Hippuric acid	36	59
7	Tryptophan	7	23	27	Quinolinic acid	0	4
8	Tryptamine	65	42	28	Picolinic acid	8	25
9	Dimethyltryptamine	57	35	29	Nicotinic acid	36	59
10	Indican	78	19	30	Nicotinuric acid	22	35
11	Indolylacetonitrile	81	94	31	Nicotinamide	68	62
12	Indoleacetamide	76	79	32	N-Methylnicotinamide	71	65
13	Indoleacetaldehyde	Decomposes		33	Quinaldic acid	25	48
14	5-Hydroxyindole	83	82	34	Kynurenic acid	46	34
15	5-Hydroxyindoleacetic acid	17	58	35	Xanthurenic acid	11	22
16	5-Hydroxytryptophan	6	12	36	Isatin	77	76
17	5-Hydroxytryptamine	58	21	37	Uric acid	4	0
18	Bufotenin	49	17	38	Citrulline	1	7
19	Anthranilic acid	33	88	39	Urea	52	50
20	*p*-Aminobenzoic acid	22	83	40	Riboflavin	21	30

Table IV gives the hR_F values of forty tryptophan metabolites and related compounds on thin layers of Silica Gel GF_{254} using Solvent A for development in the first dimension and Solvent B for development in the second dimension. Fig. 3 shows the actual sizes and shapes of the spots of a mixture of the thirty-two compounds separated by means of the above solvent system. In the past it has not been possible to achieve this on a single thin-layer chromatogram. Fig. 4 is an example of a thin-layer chromatogram obtained by this method for a pathological urine. The effectiveness of the saturation procedure of SANKOFF AND SOURKES has been verified and the necessity for removal of solvent residues from the first dimension for reproducible hR_F values in the second dimension has been shown.

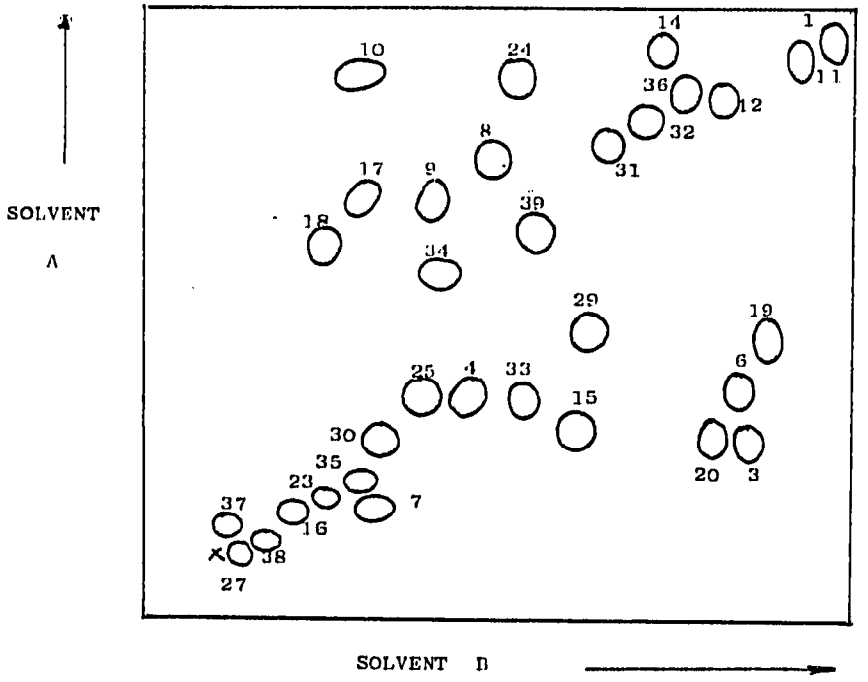

Fig. 3. Separation of tryptophan metabolites and related compounds on thin layers of Silica Gel GF_{254}. For conditions see text. For key see Table IV.

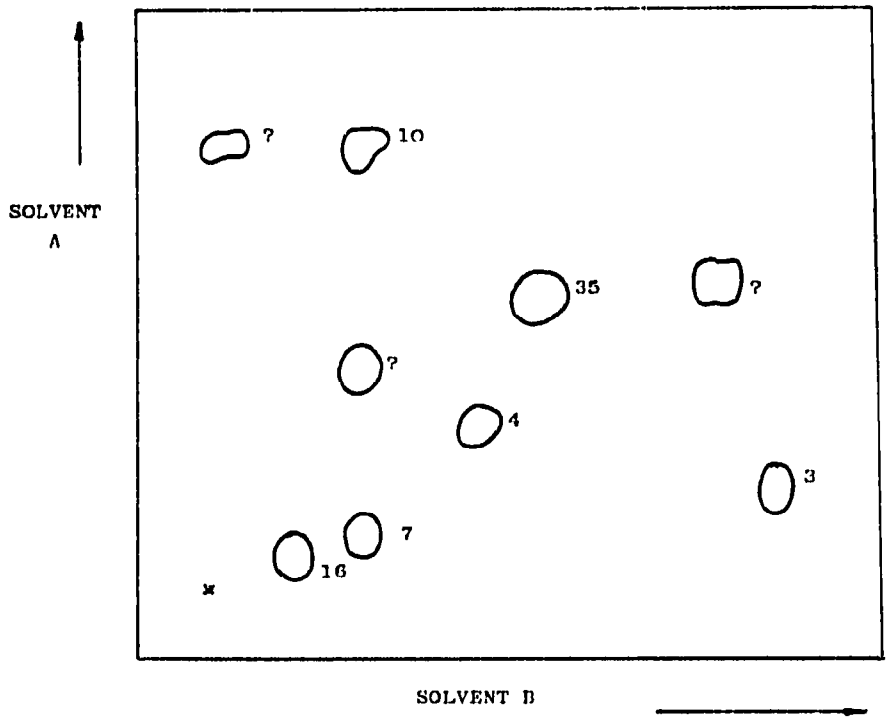

Fig. 4. Separation of tryptophan metabolites from urine of a subject suffering from phenylketonuria on thin layers of Silica Gel GF_{254}. Amount applied to layer equivalent to 1 ml untreated urine. For key see Table IV.

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