CHROMATOGRAPHY OF AMINO ACIDS, INDOLES AND IMIDAZOLES ON THIN LAYERS OF AVICEL AND CELLULOSE AND ON PAPER

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As a preliminary to the investigation of amino acids, indoles and imidazoles in the urine and blood of primates, we have investigated the thin layer and paper chromatography of these compounds. Although a great deal of work has been reported on the TLC of amino acids in chemically pure mixtures and in protein hydrolysates, little has been done on natural materials such as urine and blood and, as far as we are aware, nothing has been reported on the separation of indoles and imidazoles from such sources.

The most extensive study on the TLC of amino acids is that of VON ARX AND NEHER[1], who showed that cellulose was the best material of those then available. Subsequently BUJARD AND MAURON[2] described successful separations on cellulose and we have confirmed these separations using synthetic mixtures of amino acids. Cellulose contains a large amount of impurity which fortunately moves in the region of the solvent front and so does not interfere with the amino acids. However this material does interfere with the separation of indoles and imidazoles and it would seem essential to pre-wash the layer before chromatography. More recently, WOLFROM *et al.*[3] have suggested the use of Avirin, a low-cost micro-crystalline cellulose and Avicel which is the corresponding pharmaceutical grade. We have found Avicel to be equally valuable but much slower then cellulose; it also contains some fast moving impurities.

Synthetic indoles were examined by STAHL AND KALDEWEY[4] using silica gel and solvents other than those normally used for the paper chromatographic separation of urinary compounds. We had previously found that the standard urinary solvents described by JEPSON[5] yielded almost identical patterns when applied on silica plates and now find that similar but not identical separations can be obtained on the celluloses. Somewhat similar findings hold for the imidazoles.

APPARATUS AND METHODS

Paper chromatography was carried out as previously described by SMITH[6] using a frame holding five sheets of 10 × 10 in. Whatman No. 1 paper. Thin layer plates were prepared using the Shandon Unoplan Apparatus in which the plates are pressed up to two guide rails to give a completely level surface for spreading. The Unoplan Spreader yields margins of about 7 mm which is too wide for two-way runs and so one side was cut down to a width of 1.5 mm which then gave a margin of 2–3 mm,

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PROTEIN AND URINE AMINO ACIDS; OTHER AMINO ACIDS (R_F × 100)

P = Paper; C = cellulose MN 300; A = Avicel.

No.	Name	BuA			BuP			IPrAm			PrAm			PrA			BuAcD		
		P	C	A	P	C	A	P	C	A	P	C	A	P	C	A	P	C	A
1	Aspartic acid	19	27	21	21	31	21	1	1	0	12	17	15	14	18	14	12	14	9
2	Glutamic acid	27	41	28	25	40	33	1	2	2	14	22	16	23	37	22	9	15	9
3	Serine	23	32	25	31	32	25	7	7	7	28	30	28	20	23	15	38	43	40
4	Glycine	26	33	24	30	30	22	7	8	7	28	29	24	22	22	15	25	27	23
5	Threonine	29	38	33	36	38	36	13	14	8	38	41	41	28	32	24	55	64	54
6	Alanine	37	43	36	32	41	34	15	16	13	37	40	33	32	35	25	31	37	27
7	Valine	51	61	55	50	53	50	25	32	23	51	57	49	50	58	49	47	56	44
8	Isoleucine	65	71	68	59	62	58	35	44	36	65	65	69	60	65	60	55	62	56
9	Leucine	66	73	71	62	64	61	38	50	40	67	68	74	64	69	64	57	64	59
10	Histidine	15	23	18	27	29	23	6	7	6	28	32	28	13	13	6	34	38	33
11	Lysine	12	18	17	13	5	1	4	4	2	18	18	14	12	12	7	29	32	28
12	Arginine	16	22	18	15	7s	4	5	4	3	22	21	14	15	19	9	8	5	3
13	Phenylalanine	59	67	62	60	64	59	31	36	32	62	64	67	54	59	60	57	65	59
14	Tyrosine	43	53	47	59	62	59	15	15	12	42	47	45	40	49	44	39	48	38
15	Tryptophan	50	58	50	62	64	61	19	22	19	50	55	51	42	49	42	54	63	54
16	Proline	35	45	38	37	42	33	18	20	17	40	41	39	35	39	29	34	39	33
19	Cystine	8	13	9	17	18	13	0	1	1	13	17	13	7	6	4	14	17	10
20	Cysteic acid	10	14	12	28	36	31	1	2	1	17	21	15	10	13	9	21	26	19
21	Methionine	50	59	50	53	57	52	20	24	18	52	55	55	46	53	46	48	56	48
22	Methionine sulphoxide	20	30	23	28	31	26	7	7	6	28	31	31	19	23	16	20	22	20
23	Methionine sulphone	22	32	25	35	41	35	9	9	8	31	35	36	21	26	18	33	37	33
24	β-Alanine	40	45	39	29	33	27	10	9	6	28	30	24	30	31	21	32	37	29
25	α-Amino-*n*-butyric acid	44	53	45	42	48	41	21	24	20	44	47	41	41	45	34	40	47	37
26	γ-Amino-*n*-butyric acid	45	53	45	29	34	27	10	11	8	30	32	25	41	45	30	36	44	34
27	β-Amino-isobutyric acid	49	56	48	35	42	33	15	18	13	38	42	33	43	47	33	39	47	38
28	Asparagine	15	22	16	17	22	16	5	5	5	22	25	18	11	14	11	19s	22	19
29	Citrulline	20	28	23	25	32	23	4	4	2	22	27	20	19	20	16	17	20	12
36	Ethanolamine	35	50	37	46	12	7	53	64	57	61	58	46	46	44	29	—	64	—
30	Glutamine	23	33	22	27	33	22	4	6	4	24	31	20	21	21	14	16	23	15
31	Ornithine	12	15	15	13	6	2	3	3	5	18	16	15	10	10	5	24	26	24
32A	1-Methyl-histidine	16	21	17	29	29	25	8	9	8	31	36	33	12	12	7	24	27	22
32B	3-Methyl-histidine	21	29	21	34	36	33	6	8	6	32	37	32	17	20	10	27	31	21
33	Taurine	22	28	22	36	42	36	15	17	15	36	42	40	26	29	22	41	50	42
34	Urea	54	56	55	54	55	54	44	48	45	58	54	54	53	54	46	42	48	35
102	α-Amino-isobutyric acid	46	—	47	44	49	42	24	25	21	47	49	43	45	48	37	36	46	34
105	α-Amino-*n*-caprylic acid (α-Amino-*n*-octanoic acid)	81	89	89	75	80	78	57	73	66	84	90	87	80	88	91	67	76	73
108	α-Amino-*n*-valeric acid (Norvaline)	55	63	57	51	56	49	30	35	31	56	58	60	54	60	53	50	57	49
164	Sarcosine	30	38	31	33	36	29	15	15	15	35	35	32	28	30	23	—	35	31
142	Tyrosine, 3-iodo-	59	65	58	—	74	74	10	8	8	—	—	—	62	65	75	—	—	—

this being satisfactory for the second direction. Cellulose MN 300 was used as described by VON ARX AND NEHER[1] and Avicel as described by WOLFROM *et al.*[3] except that the layer was reduced to 300 mμ. Both cellulose and Avicel were allowed to dry overnight as better plates were so obtained. TLC was carried out in six-plate frames with 200 ml solvent in the tank at an ambient temperature of 25°. In the absence of a constant temperature room it was necessary to enclose the tanks in insulated cardboard boxes to obtain even solvent ascent. Origins were placed 2.5 cm from the lower edge of the paper and 1.5 cm from the lower edge of the glass plates. For two way runs, 30–60 min drying in the fume good was adequate and this is preferable to heat-drying as many indoles are labile under these conditions. The location reagents used were ninhydrin, dimethylaminobenzaldehyde (Ehrlich's) and diazotised sulfanilic acid as previously described by SMITH[6] and, in general, the colours were considerably more stable on plates than on paper.

R_F VALUES

Six solvents have been investigated on all three media. Butanol–acetic acid, isopropanol–ammonia and butanol–pyridine are those commonly used for the paper chromatography of the compounds being investigated, propanol–acetic acid and propanol–ammonia are variations on the first two above and butanol–acetone–diethylamine–water is that found most useful by VON ARX AND NEHER[1]. The composition and times in hours for ascent above the origins of these solvents at 25° are given in Table II; R_F values are given in Tables I, III and IV.

TABLE II

SOLVENT COMPOSITION AND HOURS FOR ASCENT

Solvent		*Paper (P) 20 cm rise*	*Avicel (A) 12 cm rise*	*Cellulose (C) 15 cm rise*
BuA	*n*-Butanol–acetic acid–water (60:15:25)	7	4.5	5
BuP	*n*-Butanol–pyridine–water (60:60:60)	7	4.5	5
IPrAm	Isopropanol–water–ammonia (0.88) (200:20:10)	8.5	4.3	5
PrA	*n*-Propanol–acetic acid 1 *N* (3:1)	7.5	4.3	5
PrAm	*n*-Propanol–ammonia 0.2 *N* (3:1)	7.5	4.3	5
BuAcD	*n*-Butanol–acetone–diethylamine–water (70:70:14:35)	4	3	2.5

R_F values were appreciably more variable on thin layers than on paper although relative migration was reasonably constant.

DISCUSSION

Each of these methods has its own advantages and disadvantages. Paper chromatography is the simplest and cheapest method but also by far the slowest. The usual time for a two-way run is about 24 h including one overnight run but, as samples are often received during the day, it is sometimes convenient to wait until all are collected and then to run overnight as no time is lost here. Cellulose is the fastest running material and two-way runs can be obtained on both cellulose and Avicel in a normal working day. However the real advantage of the thin layer method

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TABLE III

INDOLES AND RELATED COMPOUNDS (R_F × 100)

P = Paper; C = cellulose MN 300; A = Avicel.

No.	*Name*	*BuA*			*BuP*			*IPrAm*			*PrAm*			*PrA*			*BuAcD*		
		P	*C*	*A*	*P*	*C*	*A*	*P*	*C*	*A*	*P*	*C*	*A*	*P*	*C*	*A*	*P*	*C*	*A*
19	5-Hydroxy-indolylacetic acid	82	82	83	65	83	91	13	14	13	42	45	51	92	88	92	50	60	50
22	5-Hydroxy-tryptamine	57	50	52	79	65	66	61	65	66	68	63	62	53	47	43	85	85	92
24	5-Hydroxy-tryptophan	30	32	30	51	54	49	8	10	9	31	31	32	20	21	18	43	52	42
30	3-Indolyl-acetic acid	96	95	97	74	92	97	30	38	32	63	67	73	98	94	97	65	71	64
42	3-Indolyl-lactic acid	91	91	91	71	78	86	33	42	43	63	69	76	92	93	95	67	73	70
47	Indoxyl sulphate	46	48	57	82	84	95	58	64	77	76	76	89	72	66	83	83	87	89
66	Tryptamine	75	72	75	83	68	75	87	89	95	90	89	90	72	67	65	95	94	98
67	Tryptophan	50	58	50	62	64	61	19	22	19	50	55	51	42	49	42	54	63	54
71	Urea	54	56	55	54	55	54	44	48	45	58	54	54	53	54	46	42	48	35

TABLE IV

IMIDAZOLES (R_F × 100)

P = Paper; C = cellulose MN 300; A = Avicel.

No.	Name	BuA			BuP			IPrAm			PrAm			PrA			BuAcD		
		P	C	A	P	C	A	P	C	A	P	C	A	P	C	A	P	C	A
1	4-Amino-5-carboxamide imidazole	44	51	44	60	62	60	32	36	25	50	56	50	48	54	48	40	46	31
2	Anserine	15	—	15	27	24	19	7	9	7	32	37	33	8	9	6	28	32	28
3	Carnosine	15	21	15	25	22	17	4	6	4	23	28	23	8	8	5	25	33	21
4	Ergothioneine	25	33	25	39	43	39	4	5	4	28	31	28	25	28	25	13	12	8
6	Histamine	23	23	23	47	13s*	9	48	59	48	65	62	53	25s	23s	12	67	71	65
27	Histamine, β-N-acetyl-	48	56	48	79	72	69	75	84	75	71	87	86	60	61	54	65	73	62
7	Histidine	15	23	18	27	27	23	6	7	6	28	32	28	13	13	6	34	38	33
8	Histidinol	25	24	19	50	25s	16	43	55	43	65	67	61	28s	15s	9s	61	67	58
9	Hydroxymethyl imidazole	37	52	37	73	69	66	62	71	62	73	76	73	52	55	45	59	65	54
10	Imidazole	50	57	50	84	78	73	81	87	81	88	88	88	55	54	43	76	82	75
11	Imidazoleacetic acid	40	46	40	38	42	38	10	13	10	36	40	40	39	53	46	32	39	29
13	Imidazoleacrylic acid	49	53	49	52	61	62	14	15	14	34	42	42	63	67	53	38	46	32
14	Imidazolealdehyde	62	—	62	81	79	78	64	62	64	67	77	80	73	78	—	67	72	65
15	Imidazolecarboxylic acid	30	36	30	37	42	45	12	11	12	28	34	37	30	35	25	34	39	30
16	Imidazoleglycerol	28	37	28	58	57	51	29	36	29	42	52	46	36	39	24	35	39	26
17	Imidazolelactic acid	30	35	30	36	40	40	12	14	12	28	38	38	24	44	26	33	41	31
18	Imidazolepropionic acid	40	45	40	38	42	40	15	17	15	32	45	41	50	58	57	38	46	34
19	Imidazolepyruvic acid	25s	34	32	36	40s	45	6	7	6	28s	38s	37	24	35s	27s	14s	15s	40
20A	3-Methyl-histidine	21	29	21	34	36	33	6	8	6	32	37	32	17	20	10	27	31	21
20B	1-Methyl-histidine	16	21	17	29	29	25	8	9	8	31	36	33	12	12	7	24	27	22
26	4-Ureido-imidazole-5-carboxylic acid	27	28s	27	40	50	50	10	10	10	25	35	41	26s	27s	32	40	48	37
32	Acetyl histidine	36	45	36	36	45	44	13	17	13	30	40	40	30	35	31	34	43	29
33	Acetyl histidinol	44	53	44	73	70	60	67	72	67	67	81	79	51	53	48	57	65	58
34	4-Amino-imidazole-5-carboxylic acid	33	39	33	61	63	57	32	35	32	44	52	49	41	43	39	40	45	31
	Imidazole-4,5-carboxylic acid	32	38	44	60	68	77	5	3	—	35	27s	49	37	45	44	16	21	19
	1,4-Dimethyl-histidine	28	38	28	49	48	62	52	63	52	68	68	60	30	24	15s	65	69	64
	1-Histidinol phosphate ester	14	15	14	13	19	27	0	0	0	9	8	12	9	8	4	9	10	8

* s = Streaking.

is that smaller quantities can be used and these yield much smaller spots with a consequence of better separation.

With mixtures of synthetic compounds, two-way separations show good correspondence with maps plotted from R_F values. With natural materials this is not so. All three media are sensitive to inorganic salts and the urine requires desalting prior to an examination for amino acids and imidazoles; indoles can be chromatographed on paper without a prior desalting but much less satisfactory results are obtained with thin layers which seem to be more sensitive to the salt. Further, although a smaller quantity is applied to the thin layers, the urea interferes more than it does on paper and, particularly with urines from non-human primates, may adversely affect the whole separation such that R_F values of the located compounds may show no correspondence with the theoretical figures. Fortunately with human urine this gives no cause for concern as the appearance of any indole (except for traces of tryptophan) on the chromatogram suggests an abnormality which must be one of three easily distinguishable diseases. However, it might be wiser to remove the urea in other cases prior to chromatography. Similarly urea interferes with the chromatography of imidazoles on thin layers when desalted urine is used.

The results of this study have been applied to an investigation of the indoles, imidazoles and amino acids in the urine and blood of a variety of primates. Although this will be reported elsewhere, a few general remarks may be made here. For indoles, we conclude that the best of the three methods is the original one described by JEPSON[5] for paper chromatography, as whole urine still containing urea and salts can be run directly. Many primate urines contain up to ten indoles (Ehrlich reactors) some of which are lost on desalting whilst others are chemically altered (hydrogenated) during this process, and consequently an untrue pattern is obtained from desalted urine. The separation of imidazoles is also not quite satisfactory for two reasons. Many urines contain substances which react with the reagent but are extractable with ethyl acetate and, presumably, are not imidazoles; a fact which seems to be confirmed by their colour reactions with the reagent. Furthermore, some urines contain imidazolepropionic acid after electrolytic desalting but not after ion-exchange desalting although the latter procedure also affects some compounds as ammonia is used to elute them. Amino acids seem to respond best after electrolytic desalting as the urea moves out of the area occupied by these compounds and satisfactory results may be obtained on paper and thin layers. With blood it is first necessary to autoclave the spot, as described by EFRON[7], and only the paper method has proved satisfactory in our hands.

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SUMMARY

A comparative study of the chromatography of some seventy amino acids, indoles and imidazoles has been made on paper and thin layers of Avicel and cellulose. The relative merits of these methods have been discussed in relation to two-way separations of these compounds occurring in pure solution and in urine and blood.

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