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AN IMPROVED TECHNIQUE FOR THE ANALYSIS OF AMINO ACIDS AND RELATED COMPOUNDS ON THIN LAYERS OF CELLULOSE

PART IV. THE QUANTITATIVE DETERMINATION OF AMINO ACIDS IN URINE

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SUMMARY

A new method is described for the accurate quantitative determination of amino acids in urine using thin-layer chromatography. Interfering salts and peptides are first removed from the urine by passing it through a column of an ion-retardation resin. The urine is then chromatographed on thin layers of cellulose and the amino acids are determined quantitatively by the method of HEATHCOTE AND HAWORTH.

Several normal and pathological urines have been examined by this technique and quantitative recovery of standard amino acids has been obtained. The results obtained with individual urine samples agree well with those determined by the automatic ion-exchange method of analysis. The method seems to hold considerable promise for the analysis of amino acids in urine.

INTRODUCTION

Thin-layer chromatography (TLC) is potentially the most simple and rapid method for the analysis of amino acids in urine.

Unfortunately, when the method is applied to unmodified urine, even using the improved separation techniques of HAWORTH AND HEATHCOTE[1,2] (Parts I and II of this series), distorted chromatograms are obtained. This makes the precise quantitative determination of each amino acid impossible.

We have found this distortion to be caused not only by the presence of inorganic salts but also by the presence of oligopeptides of a basic nature[3] which markedly distort the final pattern on the thin-layer plate. We have recently carried out a survey of available desalting techniques[3] and have found that, though these remove the inorganic salts, none of them results in a completely clear pattern because of the failure to remove the basic peptides. If it were possible to overcome this difficulty, then the advantages afforded by TLC would make it automatically the method of choice for the analysis of amino acids in urine.

We have accordingly developed a new method of treatment of the urine samples which effectively removes these peptides as well as inorganic salts and which enables a clear distortion-free chromatogram to be produced.

The de-salting step consists essentially in passing the urine through a column of ion-retardation resin under standard, specified conditions.

TLC on cellulose may now be applied both to the screening of large numbers of urine samples, which at present cannot be carried out by ion-exchange methods and to the quantitative analysis of a selected number of samples.

MATERIALS AND EQUIPMENT

Urine samples

Several normal and pathological urine samples were obtained from children of both sexes after a period of overnight fasting. The samples were desalted as soon as possible after arrival in the laboratory.

Ion-retardation resin

The ion-retardation resin used was Bio-Rad AG11A8 50–100 mesh (Batch No. 5145-16 B-2198, obtained from Calbiochem)*.

Cellulose powder

The cellulose powder used in this investigation was MN-300 (without binder)**; it was washed by the technique described previously in Part I of this series[1].

*Solvents for chromatographic development****

The 2-methylbutanol-2 was of G.P.R. grade and the butanone and propanone of M.F.C. grade. All other solvents were of Analar grade.

Detections reagents

Ninhydrin–cadmium acetate (0.2% w/v) reagent was used for the detection and determination of amino acids and isatin–cadmium acetate (0.2% w/v) for the imino acids. These were prepared as described previously[2].

Chromatographic equipment

Shandon§ equipment was used throughout this work for the preparation and development of the cellulose thin layers.

Chromatographic columns for desalting were prepared from glass tubing (1.5 × 30 cm). Indentations were made about 1 cm from one end in order to support a glass wool plug which in turn supported the ion-retardation resin. The flow rate was controlled by means of a Hoffmann screw clip attached to a length (2 cm) of rubber tube at the base of the column.

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* Calbiochem Ltd., 10, Wyndham Place, London W.1.
** Macherey, Nagel and Co. Ltd., Agents Camlab (Glass) Ltd., Cambridge.
*** Hopkins & Williams Ltd., Freshwater Road, Chadwell Heath, Essex.
§ Shandon Scientific Co., 65 Pound Lane, London N.W.10.

Densitometer

The instrument used was the Joyce Loebl "Chromoscan" double beam densitometer with thin-layer attachment and the reflectance mode of operation was used throughout this work.

METHODS

Preparation of desalting column

The column was prepared from an aqueous suspension of Bio-Rad AG11A8 to the required dimensions (1.5 × 12.5 cm). The resin was then washed with distilled water (100 ml), to remove impurities.

Operation of desalting column

After removal of excess water from the surface of the resin the urine sample (2 ml) was applied carefully to the inside of the tube by means of a pipette so that the surface of the resin was not disturbed. The urine was then washed onto the column with distilled water (1 ml) which was allowed to pass through the column until the resin surface was again just free from water. The amino acids and related compounds were eluted from the resin with distilled water (19 ml) at a flow rate of about 2 ml/min.

The first 13 ml of the eluate was discarded and the next 6 ml, which contained the amino acids, was collected.

In order to prepare the column for re-use it was then washed with distilled water (100 ml) at a fast flow rate (10 ml/min).

Preparation of thin layers

Thin layers of cellulose, initially 400 μ thick, were spread on 20 × 20 cm glass plates as described previously by HAWORTH AND HEATHCOTE[1].

Development of thin-layer chromatograms[1]

The amount of desalted, peptide-free urine applied to the thin layer varied with the concentration of amino acids in the urine but 20 to 40 μl was usually sufficient with most urines to show about 15 different amino acids. It is important to keep the spot size as small as possible for successful chromatography and for this reason samples were applied using capillary pipettes (5 μl) with intermediate drying in a stream of warm air. The solvent systems used were 2-propanol–butanone–1 *N* hydrochloric acid (60:15:25, v/v) for development in the first dimension and 2-methyl-butanol-2–butanone–propanone–methanol–water–(0.88) ammonia (50:20:10:5:15:5, v/v) for development in the second dimension. The chromatograms were developed in the first dimension until the solvent front was 13 cm from the origin (2.5 h). Then the plates were removed from the tank, dried in a stream of cool air for 15 min and heated in a convection oven at 60° for 15 min to remove final traces of solvent. Rapid cooling was effected by standing the plate in a current of cold air for 5 min.

When cool, the chromatograms were developed in the second dimension, at right angles to the first dimension, until the solvent front had again reached 13 cm from the origin. The solvent was removed by heating the plate in a convection oven at 60° for 15 min.

Detection of amino acids. When cool, the plates were sprayed with the ninhydrin–cadmium acetate chromogenic reagent until they appeared translucent. After heating at 60° for 15 min in the convection oven, the treated plates were allowed to stand in a dark, ammonia-free atmosphere for 4 h.

During this time the coloured amino acid complexes develop their strongest and most consistent colour value[2]. For the detection of proline and hydroxyproline the plates were similarly treated with isatin–cadmium acetate chromogenic reagent. This was followed by heating at 90° for 10 min and allowing them to stand for 1 h. A separate plate was used for the detection of proline and hydroxyproline since we have found this to give more consistent results[2].

Densitometry. In the case of the amino acid spots stained red by ninhydrin–cadmium acetate reagent, the filter used was 490 nm in wavelength while the spots staining blue by isatin–cadmium acetate reagent were scanned at 620 nm. The area under the densitometric curve was measured using the relationship

$$\text{area} = \text{peak height} \times \text{width at half height.}$$

The value for the area obtained was then related to the amount of amino acid present by reading from prepared standard graphs for each amino acid as described in ref. 2.

TABLE I

AMINO ACIDS EXCRETED BY NORMAL SUBJECTS

Amino acid	*Concentration (μg/ml of urine)*							
	1		*2*		*3*		*4*	
	TLC	*Ion exchange*	*TLC*	*Ion exchange*	*TLC*	*Ion exchange*	*TLC*	*Ion exchange*
Alanine	22.9	23.3	36.0	37.5	51.0	52.0	60.0	62.2
Arginine	7.5	7.6	14.2	14.0	12.2	12.0	12.0	13.0
Aspartic acid	3.8	4.1	4.1	4.0	2.0	2.2	4.4	4.7
Cysteic acid[a]	72.0	71.0	46.0	52.0	80.8	87.5	74.0	72.2
Glutamic acid[b]	23.0	24.4	7.4	8.7	2.0	1.8	3.6	3.8
Glutamine	105.3	116.7	82.4	87.0	99.7	107.0	73.0	78.0
Glycine	180.0	177.1	135.0	140.0	150.0	157.0	142.0	151.0
Histidine	132.0	130.0	112.0	115.0	138.0	141.0	200.0	201.0
Homocystine	0	0	0	0	0	0	0	0
Isoleucine	25.7	27.1	16.0	15.3	7.2	7.3	29.9	32.0
Leucine	17.4	17.1	9.1	10.0	10.9	10.7	23.7	25.0
Lysine	51.0	54.3	8.2	8.0	25.0	24.6	35.1	34.0
Methionine	2.7	3.0	0.7	0.8	1.4	1.0	2.9	3.0
Ornithine	5.7	6.2	0	0	0	0	13.9	14.0
Phenylalanine	18.6	17.9	18.0	18.7	25.2	24.0	53.1	51.0
Proline	12.1	12.2	3.7	4.0	5.5	6.0	1.4	1.4
Serine	40.1	35.2	18.8	19.4	24.2	26.0	18.2	18.0
Threonine	24.2	24.6	10.9	11.3	16.3	17.0	13.1	14.0
Tryptophan	2.8	2.9	1.8	1.8	6.7	6.7	3.6	3.7
Tyrosine	17.8	18.1	26.8	28.7	19.4	20.0	75.0	74.0
Valine	5.0	4.7	3.1	3.3	1.8	1.8	11.1	11.0

[a] The separate values obtained for cysteine, cysteic acid and cystine have been combined as "cysteic acid".

[b] Phenylacetylglutamine is not separated from glutamic acid by either technique and is therefore estimated as such.

TABLE II

AMINO ACIDS EXCRETED BY PATHOLOGICAL SUBJECTS

Amino acid	*Concentration (μg/ml of urine)*							
	Homocystinuria		*Generalized amino aciduria*		*Phenylketonuria*		*Lysinuria*	
	TLC	*Ion exchange*	*TLC*	*Ion exchange*	*TLC*	*Ion exchange*	*TLC*	*Ion exchange*
Alanine	62.0	59.1	40.5	44.1	3.2	3.1	19.5	17.1
Arginine	0	0.7	15.7	15.1	0	0	156.0	142.0
Aspartic acid	0	0.2	12.5	12.6	0	0	0	0
Cysteic acid[a]	33.9	23.2	44.6	30.1	0	0	37.5	42.0
Glutamic acid[b]	3.0	2.7	13.5	12.9	275	280	0	0
Glutamine	0	0	88.5	92.1	0	0	0	0
Glycine	47.5	56.0	55.0	50.0	13.0	13.4	126	140
Histidine	78.0	72.0	440	405	6.4	6.6	0	0
Homocystine	10.8	11.5	0	0	0	0	0	0
Isoleucine	0	0	0	0	0	0	0	0
Leucine	5.0	4.8	4.2	4.3	12.5	12.1	15.0	14.3
Lysine	12.1	8.1	23.0	19.1	3.2	2.4	315	289
Methionine	1.4	1.2	4.8	4.6	0	0	0.2	0
Ornithine	0	0	11.7	12.1	0	0	9.8	8.2
Phenylalanine	10.0	11.4	5.5	6.0	285.0	299.0	0	0
Proline	14.0	12.2	16.4	17.8	5.1	4.2	0	0
Serine	12.5	11.7	37.0	37.7	5.2	6.2	85.5	92.0
Threonine	10.0	10.0	27.5	30.9	6.2	7.7	16.5	18.8
Tryptophan	1.1	0.8	0	0	2.7	2.9	3.5	3.0
Tyrosine	11.2	12.9	8.0	9.7	2.5	3.4	18.0	19.0
Valine	10.0	9.1	4.0	3.8	12.5	11.1	0	0

[a] The separate values obtained for cysteine, cysteic acid and cystine have been combined as "cysteic acid".

[b] Phenylacetylglutamine is not separated from glutamic acid by either technique and is therefore estimated as such.

TABLE III

RECOVERY OF ADDED AMINO ACIDS AND UREA FROM URINE

Amino acid	*% recovery*	*Amino acid*	*% recovery*
Alanine	98	Leucine	97
Arginine	99	Lysine	98
Aspartic acid	93	Methionine	96
Cysteine	98	Phenylalanine	99
Glutamic acid	91	Proline	100
Glutamine	95	Serine	97
Glycine	101	Threonine	99
Histidine	93	Tyrosine	91
Isoleucine	98	Valine	97
Taurine	13	Urea	5

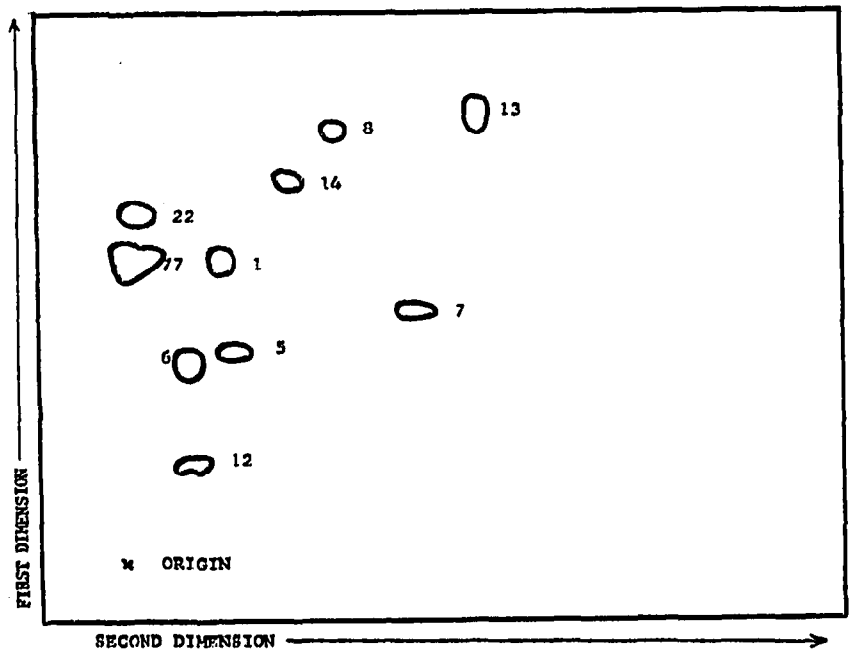

Fig. 1. Separation of amino acids in urine of phenylketonuric subject. The amino acids are numbered in accordance with Parts I and III of this series. 1 = alanine; 5 = serine; 6 = glycine; 7 = threonine; 8 = valine; 12 = lysine; 13 = phenylalanine; 14 = tyrosine; 22 = α-aminoadipic acid; 77 = phenylacetyl glutamine.

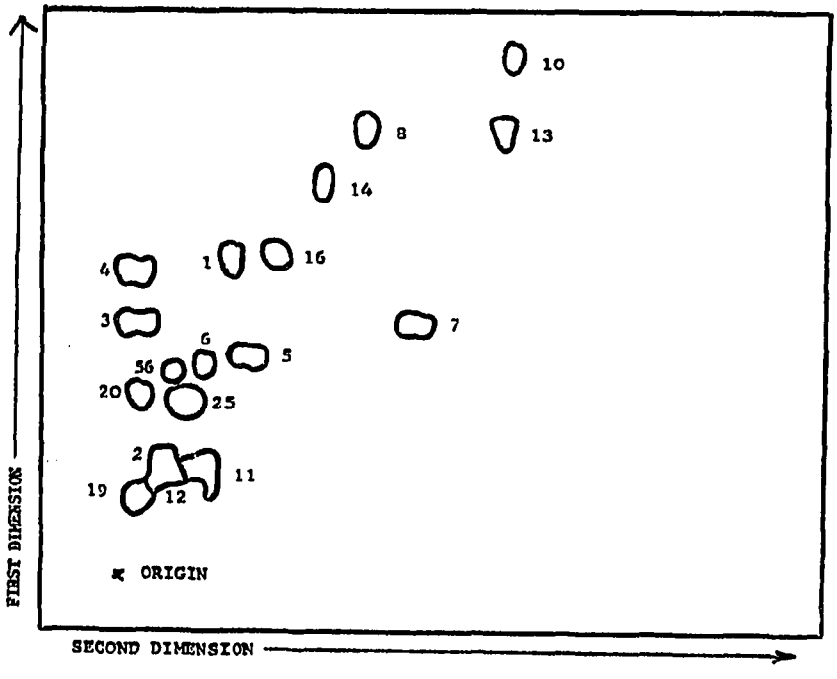

Fig. 2. Separation of amino acids in urine of subject with generalized hyperaminoaciduria. The amino acids are numbered in accordance with Parts I and III of this series. 1 = alanine; 2 = arginine; 3 = aspartic acid; 4 = glutamic acid; 5 = serine; 6 = glycine; 7 = threonine; 8 = valine; 10 = leucine; 11 = histidine; 12 = lysine; 13 = phenylalanine; 14 = tyrosine; 16 = proline; 19 = cystine; 20 = cysteic acid; 25 = glutamine; 56 = citrulline.

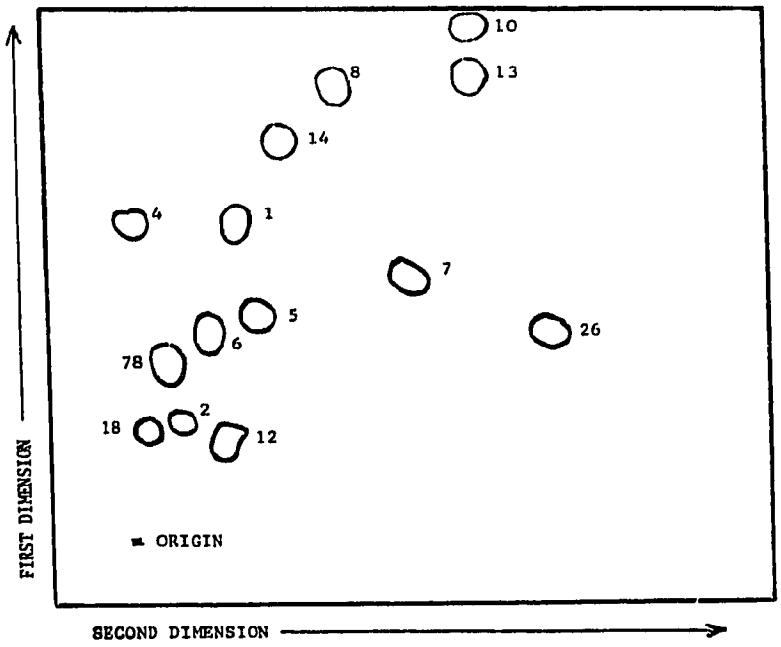

Fig. 3. Separation of amino acids in urine of homocystinuric subject. The amino acids are numbered in accordance with Parts I and III of this series. 1 = alanine; 2 = arginine; 4 = glutamic acid; 5 = serine; 6 = glycine; 7 = threonine; 8 = valine; 10 = leucine; 12 = lysine; 13 = phenylalanine; 14 = tyrosine; 18 = cysteine; 26 = ethanolamine; 78 = homocystine.

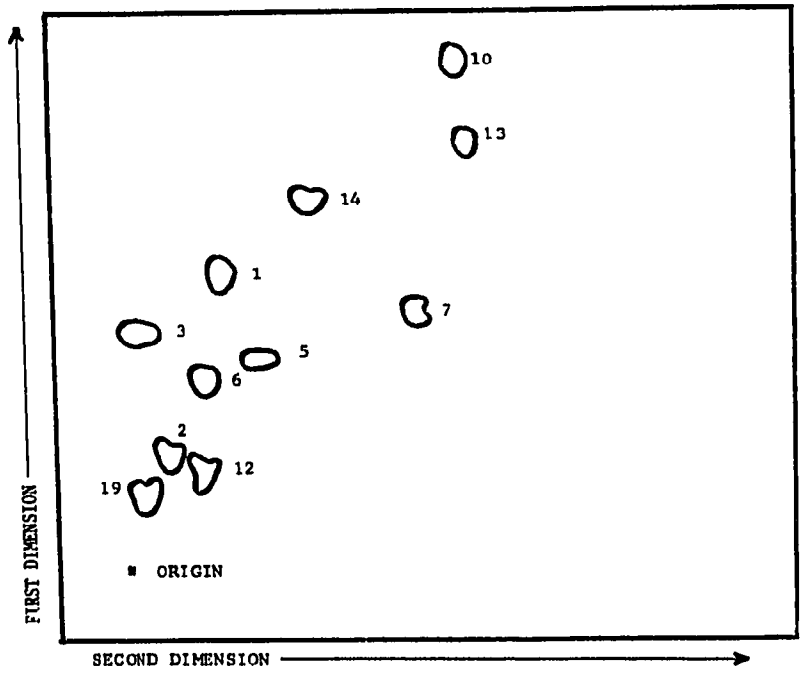

Fig. 4. Separation of amino acids in urine of lysinuric subject. The amino acids are numbered in accordance with Parts I and III of this series. 1 = alanine; 2 = arginine; 3 = aspartic acid; 5 = serine; 6 = glycine; 7 = threonine; 10 = leucine; 12 = lysine; 13 = phenylalanine; 14 = tyrosine; 19 = cystine.

RESULTS AND DISCUSSION

This investigation has resulted in a method which is highly suited to the quantitative determination of amino acids in urine samples. The urines of four normal and four pathological subjects have been examined by this procedure and by the automatic ion-exchange technique (Technicon). It is noted that the results obtained by the present thin-layer procedure agree well with those obtained by the standard column method (Tables I and II). The recoveries of the amino acids, of taurine, and of urea, have been determined by adding known amounts of these compounds to the urine samples. After desalting, the recoveries of the amino acids were found to be quantitative but those of taurine and urea were extremely low (Table III). Both of these latter compounds were almost completely removed by the desalting procedure but this removal enabled all the other amino acids to be identified unambiguously. Examples of the thin-layer chromatograms obtained for normal and pathological urines by this method are shown in Figs. 1–4.

It is important to note that the initial volume of eluate from the desalting column which is discarded contains the interfering basic peptides. That is why this initial volume is accurately measured for the column used. The conditions described here are those which apply to the exact volume of resin and flow rate given above. If the volume of the resin bed or the flow rate were altered from the given conditions it would be necessary to recalibrate the individual column.

The efficiency of the resin is impaired after about fifty samples have been passed through the column. This is due to colloidal material, present in the urine which accumulates on the column. It is therefore necessary to repack the column from time to time after washing the resin in a beaker with distilled water (3 × 100 ml).

Although the method described in this paper has been developed for the quantitative determination of amino acids in urine samples, more frequently only qualitative screening is required. For such unambiguous screening of samples it is possible to develop the ninhydrin colour immediately after chromatography. In this way large numbers of urine samples may be examined by a single operator during one working day.

REFERENCES

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