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THIN-LAYER CHROMATOGRAPHY IN THE EXAMINATION OF AMINO ACID METABOLISM AND RENAL FUNCTION

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SUMMARY

This paper shows that thin-layer chromatographic methods can be successfully applied to the analysis of a variety of biological fluids provided that adequate pre-treatment is applied so as to remove interfering peptides. It also demonstrates how amino acid metabolism can be studied directly by the determination of amino acid excretion and of true "urea clearance" by thin-layer chromatography.

AMINO ACID DETERMINATION BY THIN-LAYER CHROMATOGRAPHY

Application to mammalian urine

Over the last 20 years, it has gradually become clear that a study of amino acid excretion in urine can be most rewarding from a clinical point of view. The pioneering work of DENT and his collaborators[1,2] in using paper chromatography, in association with other methods such as polarography and microbiological assay, for the elucidation of the aetiology of cystinuria, quickly led to the discovery of new inborn errors of metabolism. More recently, of course, the MOORE AND STEIN automatic ion-exchange method has contributed much to the accurate and reliable analysis of amino acids in urine. In this method, within reasonable limits, the interference from salts and urea is minimal, and although the effects of peptides on the ion-exchange analysis of amino acids have not been studied extensively, there is no doubt that the MOORE AND STEIN automatic technique is the method of choice for the quantitative analysis of amino acids in urine.

Recently, thin-layer chromatography (TLC) has been applied to the examination of urine and has proved extremely useful for detecting gross amino acidurias. Until now, it has not been possible to obtain a clear picture of the amino acid composition by this rapid method because of the gross distortion caused by impurities in the urine. However, conventional de-salting techniques did not seem to improve the pattern much, as the chief deforming constituents of the urine appear to be peptides, which in particular distort the region of the plate that is occupied by the basic amino acids in most solvent systems. This difficulty in obtaining a clear qualitative pattern of the amino acids in urine by TLC has, until recently, prevented the application of the quantitative TLC method[3] of amino acid analysis to urine.

Over the past few years, we have developed methods at Salford for the removal of these interfering peptides from urine and other biological fluids, which have involved the use of an ion-retardation resin. The pattern of amino acid separation that has

resulted from such treatment has enabled the quantitative analysis of the amino acid to be carried out in urine in the same way as for protein hydrolysates[3]. The method of treatment of urine has been described in detail earlier[4] and a comparison with other methods has been reported elsewhere[5]. Briefly, the method consists in passing the urine over a column of Bio-Rad 11 A8 that has been previously well washed with water. The amino acids are collected practically quantitatively in a measured volume of fluid, a portion of which is then applied on to a cellulose thin layer. The improvement in the TLC pattern is clearly illustrated by the chromatograms (Figs. 1 and 2) that have been developed in the solvent systems used for quantitative amino acid analysis by TLC and devised by HEATHCOTE AND HAWORTH[3]. De-salting techniques alone, such as ion-exchange chromatography, however, produced little change in the over-all pattern. After staining with cadmium acetate–ninhydrin reagent, the individual spots were estimated by direct reflectance densitometry using the Joyce-Loebl Chromoscan instrument. The quantitative results obtained by TLC in controlled experiments agreed satisfactorily with those obtained by the ion-exchange method of analysis. A further advantage of the Bio-Rad treatment is that taurine and urea do not complicate the pattern as they are almost completely removed by elution from the column after the amino acids but before the peptides.

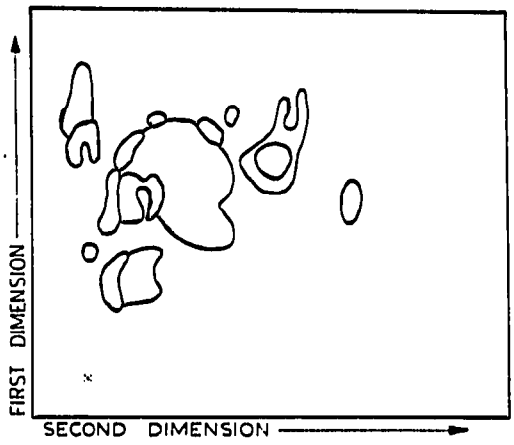

Fig. 1. Chromatogram of untreated urine (15 μl) showing interference from peptides and urea.

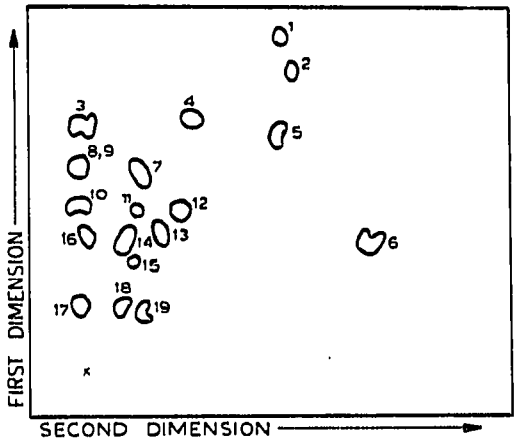

Fig. 2. Chromatogram of urine (15 μl) after treatment with Bio-Rad AG 11 A8. The amino acids are numbered as follows: 1 = Leucine; 2 = phenylalanine; 3 = α-aminoadipic acid; 4 = tyrosine; 5 = urea; 6 = ethanolamine; 7 = alanine; 8 = glutamic acid; 9 = phenylacetylglutamine; 10 = aspartic acid; 11 = β-alanine; 12 = taurine; 13 = serine; 14 = glycine; 15 = glutamine; 16 = cysteic acid; 17 = cysteine; 18 = lysine; 19 = histidine.

Application to the free amino acid pools of mould mycelia

Other biological fluids, such as culture filtrates of moulds and bacteria, often contain interfering peptides, as well as salts, which distort the amino acid pattern in both paper and thin-layer chromatography. We have applied the method of treatment using Bio-Rad AG 11 A8 to the free amino acid pools of the mycelia of a few species of moulds[6] with similar improvement. For example, Figs. 3 and 4 show the improvement in amino acid pattern obtained following the treatment of a culture filtrate of an aflatoxin-producing species of *Aspergillus flavus* with the ion-retardation resin.

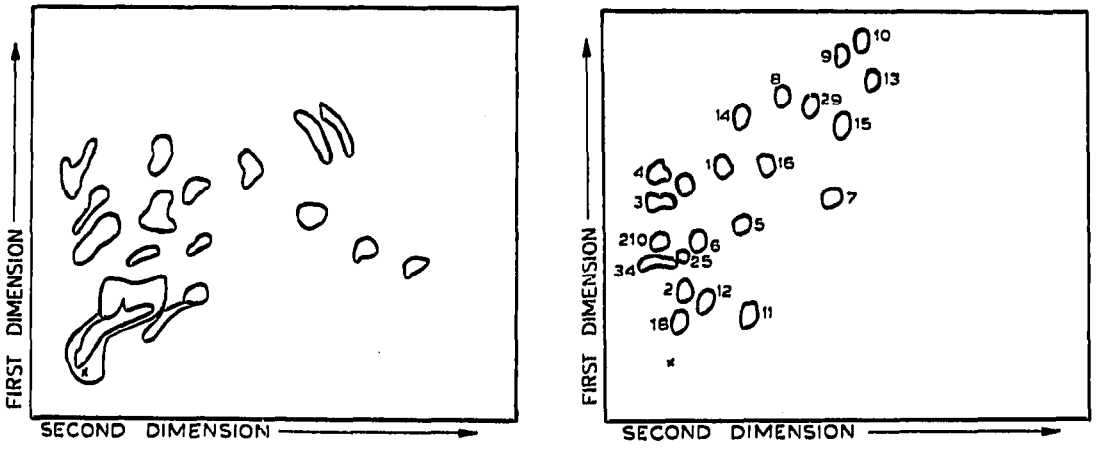

Fig. 3. Separation of untreated free amino acid pool from *Aspergillus flavus*.

Fig. 4. Separation of free amino acid pool in *Aspergillus flavus* after Bio-Rad treatment. The amino acids are numbered as follows: 1 = Alanine; 2 = arginine; 3 = aspartic acid; 4 = glutamic acid; 5 = serine; 6 = glycine; 7 = threonine; 8 = valine; 9 = isoleucine; 10 = leucine; 11 = histidine; 12 = lysine; 13 = phenylalanine; 14 = tyrosine; 15 = tryptophan; 16 = proline; 18 = cysteine; 20 = cysteic acid; 25 = glutamine; 29 = methionine; 34 = arginino-succinic acid.

In our laboratory, Miss R. EALES has applied the technique to bacterial culture fluids with similar results. There is, however, a warning that should be given here. We have observed that not all Bio-Rad samples as obtained commerically from Calbiochem have been successful in carrying out the purification step and each batch should be carefully checked to see that it will produce results similar to those which we have reported.

RENAL FUNCTION

It is common practice today to assess renal function by determining the level of urea in the blood plasma and by determining the clearance by the use of some other arbitrary substance, such as inulin, mannitol or sodium thiosulphate.

The clearance of endogenous creatinine has been held to approximate to the inulin clearance and so, because it is so much easier to determine, has been fairly widely used as a measure of the glomerular filtration rate. However, there is evidence that the kidney tubules can excrete a certain amount of creatinine in man at raised blood levels and this may contribute to its removal from the blood. Creatinine may therefore be normal when appreciable increase in blood urea has occurred. Nevertheless the method seems to be still widely used, although wide discrepancies have been reported[7].

Furthermore, while the determination of blood urea concentration gives a useful indication of the degree of renal failure, it does not rise above the accepted normal maximum level until renal function is reduced by at least 50 %. It is our belief that a true assessment of renal function requires the determination of urea clearance by measurements of urea in both plasma and urine over a specified period of time. Both of these determinations can be carried out by the methods which we have recently published and which involve the use of TLC.

Furthermore, since the urea determination is much simpler than the non-protein nitrogen determination and in almost all instances gives the same information, the TLC methods that we have developed would seem to simplify the approach to renal function testing.

DETERMINATION OF UREA IN URINE BY THIN-LAYER CHROMATOGRAPHY

The method, which can be carried out on 1 μl of urine, has been fully described elsewhere[8]. It consists in applying the urine sample to a cellulose thin layer and developing in the first dimensional solvent of HEATHCOTE AND HAWORTH[3]; after staining with Ehrlich's reagent, the concentration is determined by reflectance densitometry. Table I shows how the results compare with other standard methods of urea determination.

TABLE 1

UREA CONCENTRATION IN URINE

Comparison of different methods; urea concentration in mg per 100 ml.

Urine sample	*Present study (TLC)*	*Automatic analyzer*	*Urease*
1	900	920	966
2	730	760	783
3	625	600	652
4	911	920	989
5	1124	1160	1219
6	2135	2160	2180
7	1122	1180	1212
8	943	950	988
9	1502	1510	1584
10	1219	1240	1302

DETERMINATION OF BLOOD UREA AND ITS CLEARANCE FROM PLASMA[9]

For the determination of urea in blood plasma, a 2-μl sample was applied to the cellulose layer, developed and stained as for urine. The clearance tests were carried out on ten normal adults after a period of overnight fasting. The subjects were each given 1 litre of water to drink and then allowed to rest for 1 h. After this time, the bladder was emptied, the specimen being discarded. The bladder was then emptied after a further 30 and 60 min, each sample being saved and the void volume noted. The urine samples were pooled for analysis. A sample of oxalated venous blood was drawn after 45 min, the blood cells were centrifuged off and the plasma was retained.

A 1-μl sample of the pooled urine was developed side by side with the plasma sample and urea clearance was calculated from the equation

$$\text{Clearance} = \frac{UV}{P}$$

where U and P are the concentrations of urea in urine and plasma, respectively, and V is the volume (ml) of urine excreted per minute. The results obtained agreed well with those determined by the automatic analyzer method (Technicon).

THE DETERMINATION OF AMINO ACID EXCRETION FROM AN ARBITRARILY SELECTED SAMPLE

On many occasions it is practical to collect only a single sample of urine, particularly with elderly subjects who may be adequately treated as out-patients. In such instances, it is common practice to express the amino acid excretion in terms of another urinary parameter which is chosen because it is considered to remain resonably constant. We have recently carried out a critical examination of the reliability of various commonly used parameters for this purpose over a limited range of normal subjects.

From the results in Table II, it can be seen that the most consistent reference standard against which to express the amino acid excretion is urea nitrogen, with a mean coefficient of variation of about 19 %. The total nitrogen figure was also found, in these experiments, to be almost as reliable with an average mean coefficient of variation of 20 %. Other parameters show an occasional mean coefficient of variation that is approximately of the same order but urea nitrogen (and to a slightly less extent total nitrogen) seem to be the most consistently reliable reference standards. This would not, of course, be necessarily so in those pathological cases where the urea content of the blood and urine are abnormally high. It is perhaps worthy of note that such standards as total 24 h void and creatinine that have been much used in the past are even more unreliable than when the concentration of amino acids is expressed against unit volume of the arbitrary sample of urine.

TABLE II

COMPARISON OF MEAN COEFFICIENTS OF VARIATION IN AMINO ACID EXCRETION WHEN EXPRESSED IN TERMS OF DIFFERENT URINARY PARAMETERS

Subject	*Mean coefficient of variation* (%)				
	Per ml of void	*Per total void*	*Per gram of total nitrogen*	*Per gram of urea nitrogen*	*Per gram of creatinine*
1	41	64	22	18	68
2	44	47	20	21	54
3	19	56	26	23	25
4	40	45	20	20	75
5	21	59	18	17	56
6	41	69	15	13	50
Average	34	57	20	19	55

In conclusion, therefore, it is possible to obtain results on one TLC plate for the quantitative determination of the amino acid excretion expressed against a defined standard and also for the urea clearance. This is effected by applying one sample of urine and one of plasma for one-dimensional chromatography (urea determination) and one additional sample of urine for two-dimensional chromatography (amino acids).

The plate is then scored, after development in the first solvent system, in order to prevent development of the urea samples in the second dimension (see Fig. 5). Staining is carried out with Ehrlich's reagent for urea determination and with cadmium

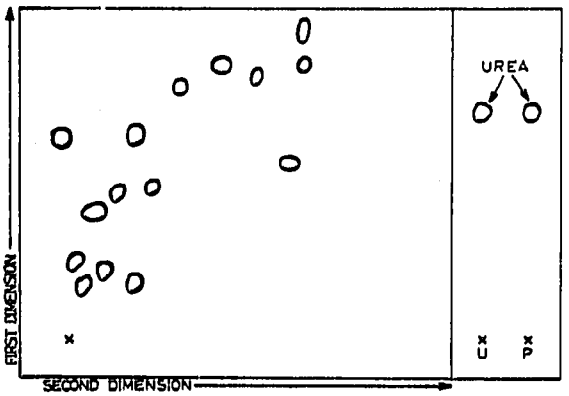

Fig. 5. Simultaneous determination of urea clearance and amino acid excretion by thin-layer chromatography. U = urine (1 μl); P = plasma (2 μl).

acetate–ninhydrin for the amino acids. Reflectance densitometry using the Joyce-Loebl Chromoscan, or another instrument, is used to determine the amounts of urea or amino acids present as described in previous papers.

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