Thin-layer chromatography of trinitrophenyl derivatives of amino acids in urine and plasma

D. PHILPOTT

From the Department of Pathology, General Hospital, Northampton

SYNOPSIS The preparation and separation by thin-layer chromatography of the trinitrophenyl derivatives of amino acids in plasma and urine is described. The semi-quantitative procedure presents a useful addition to existing methods for screening biological fluids for aminoacidopathies.

The use of 2,4,6-trinitrobenzene-1-sulphonic acid (TNBS) for the preparation of the trinitrophenyl derivatives of amino acids and peptides was described by Okuyama and Satake (1960) and Satake, Okuyama, Ohashi, and Shinoda (1960). It was observed by Nitecki, Stoltenberg, and Goodman (1967) that mixtures of trinitrophenyl-amino acids could be resolved remarkably well by thin-layer chromatography. They further noted that the preparation of these derivatives presented a number of distinct advantages over that of dinitrophenyl amino acid derivatives prepared with the aid of 1-fluoro-2,4-dinitrobenzene. The TNBS reagent is water soluble, resulting in homogeneous reaction mixtures, and side reactions are largely avoided.

The technique of Nitecki *et al* (1967) has been applied here, with some modification, to urine and plasma, the amino acid profiles being investigated densitometrically.

Materials

Analytical or 'special for chromatography' grade reagents were employed when available. The chloroform used contained 2% v/v of ethanol.

Thin-layer chromatography plates, 5 × 20 cm, and cylindrical jars, 6.5 cm diameter, approximately 22 cm high, and with ground glass lids,

were supplied by Camlab (Glass) Ltd, Cambridge.

Silica gel G¹ (Kiesel G nach Stahl) was used. Distilled water, was added to 30g silica gel in a 500-ml conical flask fitted with a rubber bung. The flask was shaken vigorously by hand for 2 min before pouring the contents immediately into a Quickfit & Quartz spreader. This quantity of slurry was sufficient to coat 20 plates with a layer 0.25 mm thick. The plates were air-dried for 24 hours before use. No activation was employed.

Methods

As changes occur in urine and plasma constituents in a few hours at room temperature, all specimens were stored at -20°C until ready for processing.

It should be established that the patient under investigation is not receiving drugs, of which metabolites in the urine would form TNP derivatives. Examples are penicillamine in the urine of patients receiving penicillin, and nitrazepam (Mogadon), which is partly excreted in a reduced form, the nitro group being converted to an amino group.

¹Anderman & Co Ltd, Battlebridge House, Tooley Street, London.

1 NEUTRAL AND ACIDIC AMINO ACID DERIVATIVES

Urine

To 1 ml of specimen in a glass-stoppered centrifuge tube was added 1 ml of water, 1 ml of 4% sodium bicarbonate solution, and a knife-point (50 mg approximately) of 2,4,6-trinitrobenzene-1-sulphuric acid. In the case of very dilute specimens, 2 ml undiluted urine was taken. The mixture was kept for two hours in the dark at 37°C, cooled, and then acidified with 1 ml 1N hydrochloric acid. The trinitrophenyl amino acid derivatives were extracted into two 5 ml portions of ether, and centrifuged briefly to assist separation. The combined extracts were dried over anhydrous sodium sulphate and then evaporated to dryness in a water bath. The residue was cooled and dissolved in 0.1 ml acetone, transferred to a small tube, and stored, well stoppered, in the dark at -20°C.

Plasma

Protein was removed by heat coagulation. To 1 ml of plasma in a small test tube was added 1 ml of 0.05N acetic acid. The mixture was heated in a boiling water bath for 3 min, cooled, and centrifuged. The supernatant fluid was decanted into a glass-stoppered centrifuge tube, subsequent processing being as described for urine.

2 BASIC AMINO ACID DERIVATIVES (URINE AND PLASMA)

Any residual ether remaining after extraction was pipetted off. Acetone, 0.5 ml, was added to the residual fluid and the mixture was saturated with solid ammonium sulphate. After centrifuging, the acetone layer was quantitatively transferred to a small tube and stored at -20°C.

CHROMATOGRAPHY

Aliquots of the acetone solutions derived from 1 (10 µl of the urine extract and 20 µl of the plasma extract) were applied with micropipettes at points 2.5 cm from the end of a 5 × 20 cm silica gel-coated plate to provide spots not exceeding 4 mm diameter and developed with 30 ml of a freshly prepared 9:1 v/v mixture of chloroform and glacial acetic acid in a cylindrical jar with a ground-glass lid. The solvent front was allowed to advance to a horizontal line marked on the plate 10 cm distant from the point of application. This required approximately 35 minutes. The developed plate was allowed to dry in the draught of a fume cupboard for 10 minutes.

Portions, 20 µl, of the acetone solutions, derived from 2 (plasma or urine), were similarly chromatographed in a freshly prepared solvent mixture composed of 30 ml n-butanol and 1 ml

1N hydrochloride acid. This took approximately 120 minutes.

Whereas all trinitrophenyl amino acid derivatives were visible initially as yellow spots on the plate, darkening was observed in varying degree on exposure to light and air, particularly in the case of the sulphur-containing amino acids. The procedures were therefore executed throughout in subdued light and densitometry was undertaken without delay after drying the plates.

DENSITOMETRY

Recording of the developed plates was carried out with a Vitatron densitometer employing an OB1 Chance blue filter and 3 × 0.2 mm slit. A pinpoint size hole made in the gel at the point of application of the extract was recorded in the tracing as a narrow rectangular trough, as was also the line marking the solvent front, thus enabling exact measurement of R_f values. When one or more derivatives were found present in excess amounts more accurate values were obtained by repeating the chromatogram with smaller aliquots of the acetone solution.

Results

The excellent separation of mixtures of trinitrophenyl derivatives of amino acids obtained by Nitecki *et al* (1967) by thin-layer chromatography with pure solutions was repeated when applied to urine and plasma. Nine to 10 clearly defined spots of trinitrophenyl derivatives of neutral and acidic amino acids were normally obtained and were recorded as well defined peaks on the densitometer tracings. They were invariably preceded on the plate in both urine and plasma chromatograms by the crescent-shaped spot of picramide. This showed intense yellow fluorescence under ultraviolet light at 350 mµ. Its presence has been attributed to the action of ammonia, derived from glutamine, on the TNBS reagent. Urea, too, was found here to be 'hydrolysed' to some extent to produce picramide in similar fashion. Fortuitously, the picramide spot formed a useful marker to which the trinitrophenyl amino acid derivatives could be referred. They have been designated R_p values and were adopted in preference to R_f values due to their greater reproducibility. With care in the preparation of the plates (R_p × 100) values (Table I) were found to be reproducible within ± 2. Typical chromatograms of trinitrophenyl derivatives of neutral and acidic amino acids derived from urine and plasma are illustrated in Figures 1-3. The small amount of picric acid produced and extracted remained at or near the origin.

Chromatography of the ether-insoluble basic

Fig. 1 Urine, adult, showing Pic, GNH_2 , Ser, Thr, Asp, Glu, Gly, Try, Ala, Ph.Al, Leu, Picr.

Fig. 2 Plasma (child, aged 2 yr, epileptic), showing Pic, GNH_2 , Ser, Thr, Asp, Glu, Gly, Try, Ala, Ph.Al, $\alpha\text{-NH}_2\text{Bu}$, Leu, Picr.

Key to Figures 1 and 2

α -Aminobutyric acid = $\alpha\text{-NH}_2\text{Bu}$; alanine = Ala; aspartic acid = Asp; glutamic acid = Glu; glutamine = GNH_2 ; glycine = Gly; histidine = Hist; 3-methyl-histidine = 3-meth-hist; homocystine = homocyst, leucine = Leu; methionine = Met; phenylalanine; Ph.Al; picric acid = Pic; picramide = Picr.

amino acid TNP derivatives presented some difficulties. The salting-out procedure adopted here proved more satisfactory than the direct spotting of the acid reaction mixtures onto the plates proposed by the original authors. The acid-butanol solvent mixture finally adopted was the best of a wide range of solvent mixtures investigated. Figure 4 illustrates a typical chromatogram of the trinitrophenyl derivatives of basic amino acids in a normal urine. R_f values are listed in Table II.

In the case of diamino acids, where mono- and bis-substitution is possible, ether-soluble

Amino Acid	$R_f \times 100$
Citrulline	5
Glutamine	8
Cystine	9, 39
Asparagine	10
Cystathionine	14, 25
Serine	16
Threonine	21
Aspartic acid	30
Homocystine	33, 61
Glutamic acid	35
Tyrosine	38
Cysteine	47
Glycine	52
Homocysteine	62
Ornithine	63
Tryptophan	65
Alanine	78
Lysine	82
Methionine	82
Phenylalanine	86
β -Alanine	89
α -Aminobutyric acid	90
β -Aminoisobutyric acid	92
Leucine	94
Isoleucine	94
Valine	97
Kynurenine	9, 75, 85
Carnosine	85
Penicillamine	73, 89

Table I Derivatives of neutral and acidic amino acids

Amino Acid	$R_f \times 100$
Taurine	6
Ornithine	10 (broad), 83
Lysine	10 (broad), 86
3-Methyl-histidine	16
1-Methyl-histidine	21 (broad)
Arginine	37
Histidine	37 (major), 58 (minor)
Cystine	62
Homocystine	63

Table II Derivatives of basic amino acids

and ether-insoluble derivatives may be formed. Corresponding reference values are listed in the tables.

Quantitative determination of fluid amino acid content by integration of the densitometer recordings was not undertaken, as the amino acids were found not to react uniformly with the TNBS reagent. It was noted, however, that on acidification of the reaction mixture before ether extraction, fluids of normal amino acid content remained clear, or at most exhibited a slight haziness. Formation of a precipitate at this stage would indicate the presence of an

Fig. 3 Urine from a case of homocystinuria in a 11-year-old boy showing Pic, GNH_2 , Ser, Thr, Asp, Glu, Gly, Homocyst, Met, $\alpha\text{-NH}_2\text{Bu}$, Picr.

Fig. 4 Urine, adult, showing basic amino acids 3-meth-hist, Hist (two peaks).

Key to Figures 3 and 4

α -Aminobutyric acid = $\alpha\text{-NH}_2\text{Bu}$; alanine = Ala; aspartic acid = Asp; glutamic acid = Glu; glutamine; GNH_2 ; glycine = Gly; histidine = Hist; 3-methyl-histidine = 3-meth-hist; homocystine = homocyst = leucine = Leu; methionine = Met; phenylalanine = Ph.Al; picric acid = Pic; picramide = Picr.

aminoaciduria, unless a specimen of urine of high specific gravity was involved. Semiquantitative assessment for screening purposes was further provided by comparison of the densitometer tracings with those obtained from normal fluids. Normally in urine the heights of the peaks rarely exceeded that given by the picramide spot.

Conclusions

The technique described has the merit for screening purposes of speed, simplicity, and, for the most part, excellent chromatographic resolution. The use of heat coagulation for plasma protein precipitation has the advantage of being rapid but is likely to produce artefacts. However, comparison of the results with those obtained using the solvent extraction technique of Naftalin and Stephens (1965) revealed no significant differences.

One disadvantage of the method is the failure of proline and hydroxyproline to form trinitro derivatives since the reagent reacts only with the primary amino groups. Brown (1968), however, has recently recorded a derivative of proline formed on prolonged reaction in a more alkaline medium and it may prove possible to adopt the method accordingly.

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