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GAS–LIQUID CHROMATOGRAPHIC METHOD FOR THE MEASUREMENT OF METHYL UREA IN BLOOD AND URINE

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SUMMARY

A technique for the measurement of methyl urea in biological fluids is described based upon gas–liquid chromatography of its trifluoroacetyl derivative. The method requires 10 ml of either blood or urine and is capable of measuring methyl urea at concentrations of less than 5 mg/l.

INTRODUCTION

In recent years a number of reports have appeared in the literature which have been concerned with the presence of methyl urea in body fluids. These communications have dealt with its investigation as a diuretic[1], a poison[2], a normal end product of metabolism[3], and as a metabolite of the antitumour agent hydroxyurea[4]. Unfortunately all these studies have been limited by the lack of a sensitive and specific assay for methyl urea. While its identification has been possible by use of thin-layer chromatography and high-voltage electrophoresis, followed by staining with *p*-dimethylaminobenzaldehyde[5], such a procedure is not readily applied to precise quantitation.

Only one serious attempt has been made to measure accurately concentrations of methyl urea in blood[6]. This technique used ^{14}C-labelled methyl urea as a tracer for the unlabelled compound and changes in blood concentrations were calculated from changes in total radioactivity. Results obtained therefore depended upon the assumption that the label remained within the parent molecule, a supposition which is by no means necessarily true.

It is the purpose of this article to report an assay system for methyl urea in body fluids suitable for the study of its biochemistry and pharmacology.

MATERIALS AND METHODS

Silica gel M.F.C. was obtained from Hopkin and Williams (Chadwell Heath, Essex, Great Britain) and trifluoroacetic anhydride (TFAA) from BDH (Poole,

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Dorset, Great Britain). Nonanoic acid methyl ester and urease were supplied by Sigma (Kingston-upon-Thames, Surrey, Great Britain).

A solution of urease was made up by grinding tablets (Type III, 80 units per tablet) in a volume of water sufficient to give a final enzyme concentration of 80 units/ml. The theoretical rate of hydrolysis of urea by 1 ml of this solution was 34 mg/min at 30° and pH 7.0.

The gas–liquid chromatographic procedure described by Evans[7] has been applied in which separation is achieved on a 7 ft. × 3/16-in.-I.D. column of 10% polyethylene glycol adipate on Diatomite CAW, 100–120 mesh (J.J.'s Chromatography, Kings Lynn, Norfolk, Great Britain). The column was housed in a Pye Model 104 gas chromatograph (Pye Unicam, Cambridge, Great Britain), incorporating a flame ionisation detector connected to a 1-mV recorder (Leeds and Northrup, North Wales, Pa., U.S.A.) with a chart speed of 10 in./h.

Chromatography was carried out isothermally at 130° using nitrogen as the carrier gas at a flow-rate of 40 ml/min.

Procedure

Measure 10-ml volumes of blood or urine into 30-ml glass tubes and add to each 1 ml of molar phosphate buffer, pH 7.0. To tubes which contain urine add 1 ml of urease solution and to those which contain blood 0.2 ml of urease. After careful mixing incubate all tubes at 37° for 1 h. At the end of this time wash the contents of the tubes into beakers with approximately 60 ml of absolute ethanol. Bring those beakers containing urine rapidly to the boil on an electrical hot plate and then filter the hot solutions through Whatman No. 1 filter paper into 150-ml flasks. Beakers containing blood should not be treated in this way but well mixed at room temperature before also being filtered into flasks. Thoroughly wash the filter papers with more ethanol and add the washings to the filtrate.

Place the flasks onto a rotary film evaporator (Type 319, from James Jobling, Stone, Staffordshire, Great Britain, has the advantage of being able to handle four flasks at once) and reduce the volumes of the alcoholic extracts to approximately 5 ml. Wash these solutions into further 30-ml glass tubes with more alcohol and reduce them to dryness at 60° under a stream of nitrogen.

Treat each deposit with 1 ml of methanol to dissolve any soluble residue and follow with 9 ml of chloroform. Centrifuge the tubes at 1200 *g* for 5 min and gently pour the resulting supernatants onto 10-cm columns of silica gel suspended in 10% methanol in chloroform and contained in 300-mm × 10-mm-I.D. glass columns each fitted with a stopcock and sintered glass disc. Repeat the extractions and apply the resulting solutions also to the columns. Run the 20 ml of extract into the columns and discard the eluate. Now elute the methyl urea by use of 80 ml of 15% methanol in chloroform, in each case collecting the eluate into a tube immersed in water maintained at approximately 70° such that the solvent rapidly evaporates as it leaves the column.

Remove any traces of solvent which remain by use of a stream of nitrogen and treat each residue with 5–10 drops of TFAA for 2–3 min, making sure that all traces of deposit come into contact with the acetylating reagent. Blow off excess TFAA with nitrogen and dissolve the solid which remains in 1.0 ml of ethyl acetate containing 0.015% v/v nonanoic acid methyl ester as internal standard. The resulting solution is ready for injection into the chromatograph.

Prepare standards for injection by taking to dryness under nitrogen 0–2 ml volumes of a 1 g/l solution of methyl urea in methanol. Treat with TFAA and dissolve in ethyl acetate containing internal standard as for the test solutions.

RESULTS

A standard curve for methyl urea is shown in Fig. 1. It has been obtained by plotting peak height, expressed as the ratio to that of the internal standard, against concentration for solutions corresponding to blood and urine concentrations of between 0 and 200 mg/l. It can be seen that the best straight line through the points does not go precisely through the origin. This divergence, though small, has been a consistent finding and its cause has not been conclusively established. It is, however, most likely to be due to loss of methyl urea during gas chromatography, the amount of loss being constant for each injection and unrelated to concentration.

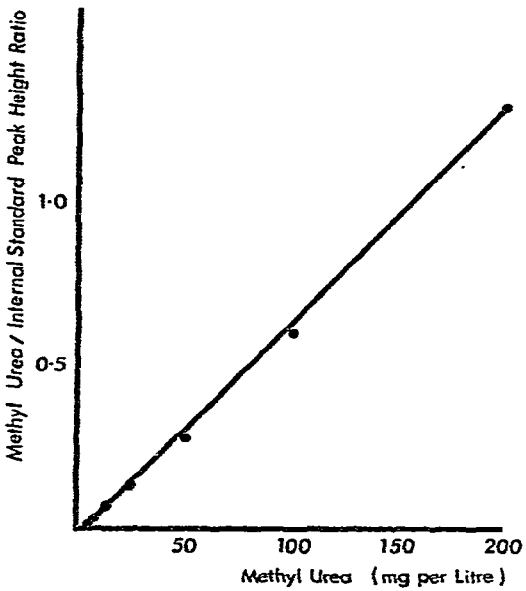

Fig. 1. Standard curve for methyl urea.

Because of this phenomenon, best results for the concentration of methyl urea in test solutions require reference to a standard curve. Less precise results can be obtained by use of the equation:

$$\text{Methyl urea concentration in blood or urine (mg/l)} = \frac{\text{Peak height ratio in test}}{\text{Peak height ratio in standard}} \times \frac{\text{Value of standard (mg/l)}}{10}$$

Investigations into the reproducibility of the chromatographic stage of the

analysis revealed a coefficient of variation of 3.1% ($N = 12$), while recoveries of methyl urea added to blood and urine at a concentration of 100 mg/l averaged for blood 96%, range 92–102% ($N = 12$), and for urine 94.5%, range 89.5–105% ($N = 12$).

Application of this method to specimens of blood and urine from twenty normal persons has in no instance revealed the presence of methyl urea at concentrations above the minimum detectable, approximately 4 mg/l under these conditions. Chromatograms typical of those obtained on normal specimens are shown in Figs. 2 and 3 with, for comparison, one obtained following the injection of 4 μl of ethyl acetate solution containing 4 μg of the methyl urea derivative (Fig. 4).

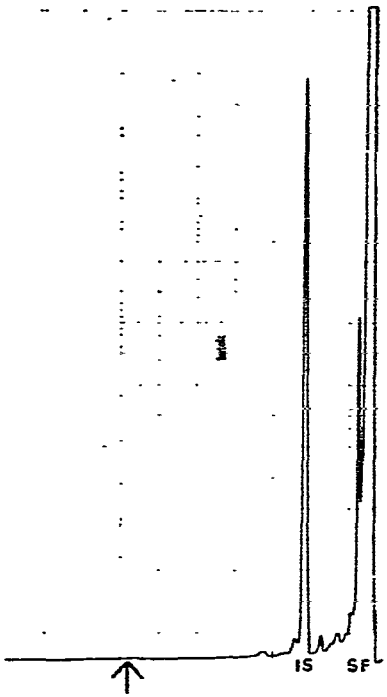

Fig. 2. Chromatogram obtained for a specimen of normal blood. SF = Solvent front; IS = internal standard. The arrow indicates the position expected to be occupied by methyl urea.

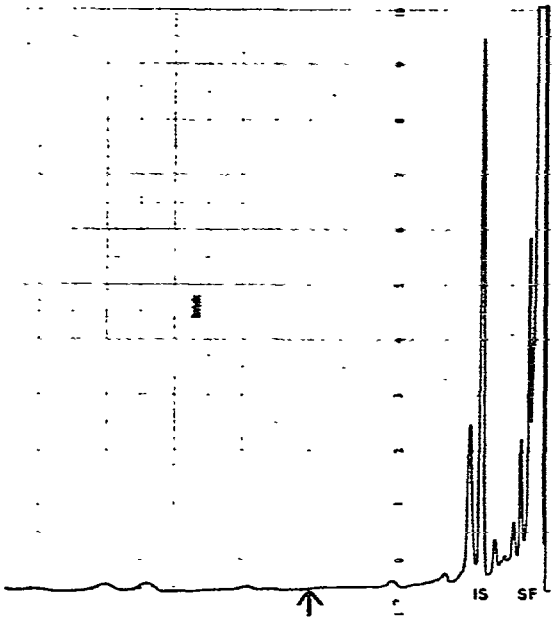

Fig. 3. Chromatogram obtained for a specimen of normal urine. SF = solvent front; IS = internal standard. The arrow indicates the position expected to be occupied by methyl urea.

DISCUSSION

Reiser[8] in 1964 was the first to apply gas–liquid chromatography to the separation of substituted ureas including methyl urea. His method did not involve the conversion of the ureas to more volatile derivatives with the result that in order to avoid serious loss of the materials which he was investigating chromatographic analyses had to be performed extremely rapidly using high carrier gas flow-rates, high temperatures and short columns. As a consequence his separations were frequently

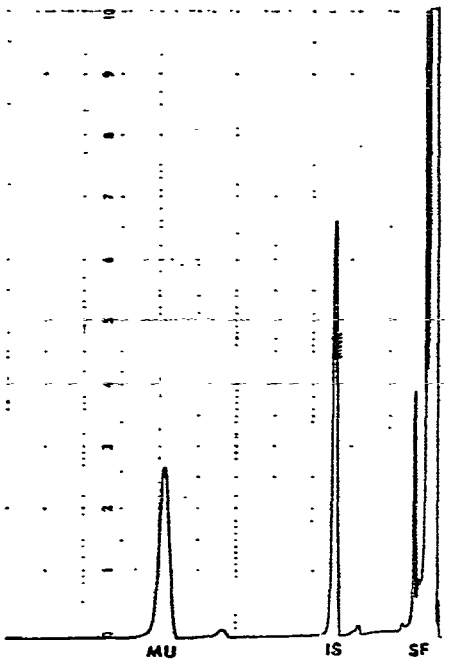

Fig. 4. Chromatogram obtained following injection of 4 μl of a standard solution containing 4 μg of methyl urea as its trifluoroacetate. SF = solvent front; IS = internal standard; MU = methyl urea.

poor, the method was insensitive and was unsuitable for quantitative measurements. The application of the trifluoroacetylation technique to methyl urea as described here has overcome many of these difficulties.

Using this technique twelve specimens can be processed ready for chromatography during the course of a normal working day. Since at the same time it would not be difficult to carry out chromatography on a further twelve specimens, the effective rate of analysis is twelve specimens per day despite the complete analysis taking nearly two days.

Methyl urea is itself extremely stable and in unpreserved blood or urine can be kept at 4° for at least two months without any signs of loss. Similarly, once having been extracted, it is stable indefinitely. Consequently, specimens can be stored for long periods of time if access to a chromatograph is limited. Once converted to its trifluoroacetate more care is required in the handling of methyl urea, since its susceptibility to breakdown in the presence of water is considerable. Stored in a desiccator away from contact with water it can be kept for many weeks, but under less stringent conditions of storage decay will begin rapidly.

Data quoted here have been based upon solution of methyl urea, following trifluoroacetylation, in 1 ml of internal standard solution. While the use of such a volume is necessary for concentrated and highly pigmented urines, smaller volumes can be used for blood and more dilute urines with a corresponding increase in sensitivity.

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