Journal of Chromatography, 149 (1978) 711–720

CHROM. 10,878

SPECIFIC METHOD FOR DETERMINING URIC ACID IN SERUM USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND GAS CHROMATOGRAPHY–MASS SPECTROMETRY

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SUMMARY

A method using a combination of high-performance liquid chromatography and stable-isotope dilution-mass spectrometry is described for the specific quantitation of uric acid in serum. The procedure involves addition of a known amount of [1,3,9-^{15}N]uric acid, as internal standard, to the serum sample followed by equilibration with the endogenous analyte. After separation from serum proteins, cationic and neutral compounds by anion-exchange chromatography, the purified uric acid is converted into its tetraethyl derivatives. High-performance liquid chromatography is used to isolate the three major isomeric derivatives for measurement of the isotope ratio *m/e* 280 to *m/e* 283. This ratio gives the relative abundances of the molecular ions of natural and of labelled tetraethyluric acid, and from it the amount of uric acid in the original serum specimen is determined.

Effective separation of tetraethyluric acid isomers can be achieved by adsorption or reversed-phase high-performance liquid chromatography using *n*-heptane–isopropanol (80:1, v/v) and methanol–water (3:2, v/v), respectively, as solvent systems.

INTRODUCTION

Uric acid is the end-product of purine metabolism in man and other primates and its measurement in human serum is most commonly used to differentiate between gouty and other types of arthritis. It is also of diagnostic value in situations where there is increased catabolism of nucleic acids, such as in the treatment of disseminated malignant disease.

Routine assays of uric acid depend upon the chemical or enzymatic oxidation of uric acid. Chemical methods, most of which are based upon the reduction of phosphotungstic acid[1], are notoriously inaccurate because other reducing substances in serum interfere in the reaction[2]. Although techniques involving uricase are more specific[1], an inter-laboratory quality control programme[3] has shown that inaccuracy is still an important problem.

In an effort to improve the accuracy of analyses in clinical chemistry, labo-

ratories in several countries are seeking to develop "definitive" methods[4], with which other methods can be compared. We are interested in developing such a method, based on stable-isotope dilution-mass spectrometry, for the assay of uric acid in serum, and we describe here a procedure from which a definitive method could be elaborated.

The procedure involves the addition to a serum sample of a known amount of an internal standard ([1,3,9-^{15}N]uric acid) in an appropriate solution. After equilibration, the uric acid is adsorbed on to an anion-exchange column, and after removal of serum proteins, cationic and neutral species, the uric acid is eluted and converted into its tetraethyl derivatives (TEUA) by reaction with alkaline diethyl sulphate. The principal isomers are isolated by high-performance liquid chromatography (HPLC) and the ratio of natural to labelled tetraethyluric acid in each isomer is measured by gas chromatography–mass spectrometry (GC–MS). Accurate estimates of the concentration of uric acid in the original sample are obtained from corresponding calibration graphs.

The present study was particularly designed for the assay of a Tentative Reference Serum which had been prepared at the Center for Disease Control, Atlanta, Ga., U.S.A.

EXPERIMENTAL

Equipment

The following equipment was used:

(1) Pye Unicam (Cambridge, Great Britain) Model 290 pH meter with a high-temperature, alkali-resistant electrode (Model HA/405-60).

(2) Waters Assoc. (Milford, Mass., U.S.A.) Model 6000A HPLC solvent delivery system and U6K universal injector.

(3) Cecil Instruments (Cambridge, Great Britain) CE 212 variable-wavelength ultraviolet monitor.

(4) Varian (Bremen, G.F.R.) 2700 gas chromatograph, coupled to a Varian-MAT 731 mass spectrometer.

(5) Pye Unicam 104 gas chromatograph with a flame-ionization detector.

Materials and reagents

Sterile liquid bovine serum was generously supplied by Dr. D. Bayse (Clinical Chemistry Division, Center for Disease Control, Atlanta, Ga., U.S.A.) in 5-ml vials, and stored at −20°.

Reference material uric acid (SRM 913) was purchased from the U.S. National Bureau of Standards (Washington, D.C., U.S.A.). A stock solution was prepared by dissolving 100 mg in lithium carbonate solution (25 ml, 54 mmole/l) and diluting to 100 ml with water; working standard solutions in the concentration range 3–5 mg/dl were made by appropriate dilution of the stock solution. [1,3,9-^{15}N]uric acid was 95% labelled and obtained from Vebberlin Chemie (Berlin, G.D.R.); a working solution was prepared by dissolving 6 mg in lithium carbonate solution (1 ml, 54 mmole/l) and diluting to 100 ml with water. All solutions were prepared on the day of use.

Chloroform, isopropanol and methanol were of analytical-reagent grade. *n*-Heptane and diethyl sulphate were of reagent grade, the *n*-heptane being re-distilled before use. These materials were obtained from BDH (Poole, Great Britain).

Dowex 2-X8 (200–400 mesh) anion-exchange resin was supplied by Sigma (London, Great Britain).

The uricase–iron reduction method[5,6] was carried out with the following reagents:

Acetate buffer. A mixture of 5 *M* sodium hydroxide solution (600 ml) and glacial acetic acid (290 ml) was adjusted to pH 4.95 and diluted to 1 l with water.

Iron(III) chloride solution. $FeCl_3 \cdot 6H_2O$ (10.8 g) was dissolved in 1 l of hydrochloric acid (36 mmole/l).

Tripyridyltriazine solution. Tripyridyltriazine (5 g) (Koch-Light, Colnbrook, Great Britain) was dissolved in 1 l of hydrochloric acid (70 mmole/l).

Colour reagent. This was prepared by mixing acetate buffer (60 ml), iron(III) chloride solution (60 ml), tripyridyltriazine solution (12 ml), methanol (48 ml) and water (192 ml).

Glycine buffer. 0.02 *M*, pH 9.2.

Uricase solution. The contents of one ampoule of uricase 500 (Hughes and Hughes, Romford, Great Britain), containing 8 units (I.U. Mahler) of the enzyme, were diluted to 1 l with glycine buffer.

Ion-exchange chromatography

A column (20 × 5.5 mm) of Dowex 2-X8 (200–400 mesh) anion-exchange resin, in the chloride form, was converted into the acetate form by washing with sodium acetate solution (1.2 mole/l) until chloride was no longer detected in the washings. The standard solution (5 ml) or Reference Serum sample (5 ml) was thoroughly mixed with a solution of [1,3,9-^{15}N]uric acid (5 ml), equilibrated at room temperature for 30 min and applied to the column. The column was washed with water (10 ml) to remove serum proteins, cationic and neutral species, and then uric acid was eluted from the column with 0.5 *M* sodium chloride solution (8 ml). In order to reduce possible inter-vial variation, the contents of four vials of Reference Serum were pooled and three 5-ml aliquots taken for analysis.

Recovery experiment. Successive 1-ml aliquots of the 0.5 *M* sodium chloride eluate were collected and the concentration of uric acid in each fraction was determined by the following differential method.

(*a*) Each aliquot was thoroughly mixed and 25 μl were incubated with glycine buffer (1.7 ml) at 45° for 5 min. Colour reagent (1.7 ml) was added and incubation continued at 45° for 5 min. The absorbance of the colour developed was measured at 595 nm against a reagent blank to give a measure of the total reducing substances in the eluate fraction.

(*b*) Non-uric acid reducing substances were measured by adding a solution of uricase (1.7 ml) to 25 μl of each eluate and incubating at 45° for 5 min to destroy any uric acid that was present. Colour reagent (1.7 ml) was then added and the mixture was incubated at 45° for a further 5 min. The absorbance was measured at 595 nm as before.

(*c*) From the difference between the two absorbance values (*a*) and (*b*), the concentration of uric acid in the aliquot was calculated from a standard graph.

Ethylation of uric acid (modification of method of Ismail and Dakin[7]). The 0.5 *M* sodium chloride eluate (8 ml) from the ion-exchange column was transferred to a 50-ml three-necked round-bottomed flask equipped with an efficient stirrer. The flask

was placed in a water-bath at 90° and 10 *M* potassium hydroxide solution (2 ml) and diethyl sulphate (5 ml) were added in quick succession. The mixture was stirred vigorously while its pH was monitored and kept above 7 by the addition of more 10 *M* potassium hydroxide solution. When all of the diethyl sulphate had reacted (5 min), a further 5 ml of diethyl sulphate were added and the addition of potassium hydroxide solution was repeated. The reaction mixture was then cooled and the tetraethyluric acid derivatives were extracted into chloroform (2 × 2 ml). The combined extract was evaporated to dryness. The residue was used for both HPLC and GC analyses.

High-performance liquid chromatography

The residue from the chloroform extract was dissolved in methanol (40 μl) for reversed-phase separation and in chloroform (40 μl) for adsorption chromatography. Reversed-phase separation was carried out on a μBondapack C_{18} column (30 cm × 4 mm I.D., Waters Assoc.) consisting of a monomolecular layer of octadecyltrichlorosilane chemically bonded to 10-μm silica. Methanol–water (3:2, v/v) was used as the mobile phase at an elution rate of 1 ml/min. Detection was by UV absorption at 240 nm.

Adsorption chromatography was performed on a 10-μm silica column (30 cm × 4 mm I.D., μPorasil, Waters Assoc.) using *n*-heptane–isopropanol (90:1, v/v) as the solvent system at an elution rate of 1 ml/min.

In order to isolate sufficient material for structural determination, a Partisil 10 silica column (25 cm × 9.4 mm; Whatman, Maidstone, Great Britain) was used for the preparative separation of tetraethyluric acids. The solvent system was *n*-heptane–isopropanol (40:1) with flow-rates of 2 ml/min for 5 min and then 3 ml/min.

Gas chromatography

The residue from the chloroform extract of the ethylation reaction was re-dissolved in chloroform (200 μl) and 2 μl were injected on to a column (9 ft. × 3/16 in. I.D.) of 3% OV-1 Gas-Chrom Q (80–100 mesh). The injector and column temperatures were 260° and 220°, respectively.

Gas chromatography–mass spectrometry

The two principal tetraethyluric acid isomers were isolated by HPLC and recovered by solvent evaporation. Each isomer was re-dissolved in chloroform (200 μl) and 2 μl of the solution were injected into the Varian gas chromatography–mass spectrometer system. The column was 3% OV-1 (6 ft. × 3/16 in. I.D.) and the carrier gas was helium (flow-rate 25 ml/min). With a column temperature of 180°, the two isomers had identical retention times. The mass spectrometer source temperature was 250°, the ionizing voltage 70 eV and the accelerating voltage 8 kV. The molecular ions of natural and labelled TEUA, *m/e* 280 and *m/e* 283, respectively, were focused at the collector by switching the accelerating voltage. The monitored ion intensities were integrated by computer and the ratio *m/e* 280 to *m/e* 283 was automatically calculated and printed out. Technical details of these procedures will be reported elsewhere.

Calibration graphs

In preliminary experiments, calibration graphs for TEUA were constructed by using standard solutions of uric acid with concentrations 0, 2, 4, 6 and 8 mg/dl and were found to be linear. Based on this finding, an alternative five-point calibration graph was constructed on the basis of a procedure suggested by Healy[8]. Two stock standard solutions were independently prepared, each with an accurately known concentration close to 100 mg/dl. Working standard solutions of nominal concentration 3.00, 4.00 and 5.00 mg/dl were prepared from one stock standard solution, and from the other, standards of 3.00 and 5.00 mg/dl. This method has the advantage of checking the accuracy of preparation of the stock solutions and of the dilution steps.

Two calibration graphs (one for each of the isomers TEUA 3 and 5; Fig. 1) were constructed from the ratio of the integrated ion intensities (*m/e* 280 to *m/e* 283) *versus* known amounts of uric acid. The concentration of uric acid in the serum was determined by algebraic interpolation from a calculated line of best fit.

RESULTS

The recovery of uric acid, as determined by the uricase–iron reduction method, was 99 ± 1% from standard uric acid solutions and 92 ± 2% from pooled human serum.

The separation of TEUA isomers by reversed-phase HPLC is shown in Fig. 1. The three major and three minor peaks detected were confirmed as TEUA isomers

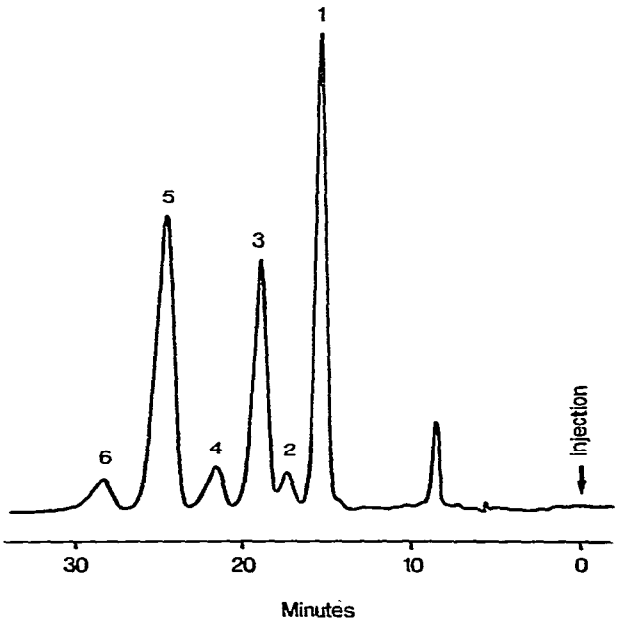


Fig. 1. HPLC separation of tetraethyluric acid (TEUA) isomers. Column, μBondapack C_{18}; solvent, methanol–water (3:2, v/v); flow-rate, 1 ml/min; detection, UV, 240 nm.

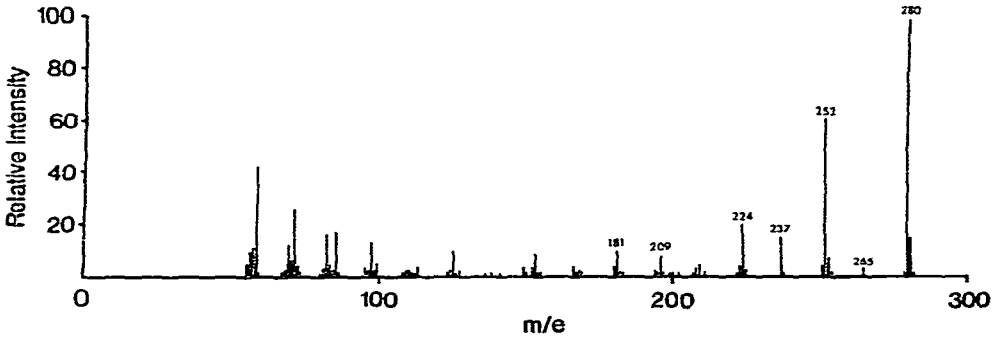


Fig. 2. Mass spectrum of TEUA 1, purified by HPLC.

by mass spectrometry. They had very similar mass spectra[7] with a prominent molecular ion at M^+ 280. The mass spectrum of TEUA 1 is shown in Fig. 2.

The separation of TEUA isomers by adsorption chromatography on a silica column using *n*-heptane–isopropanol is shown in Fig. 3. The elution of the isomers is indicated by numbers corresponding to those in Fig. 1 and it is notable that the most polar isomer, TEUA 1, which eluted first in reversed-phase chromatography, appeared as the last peak in adsorption chromatography.

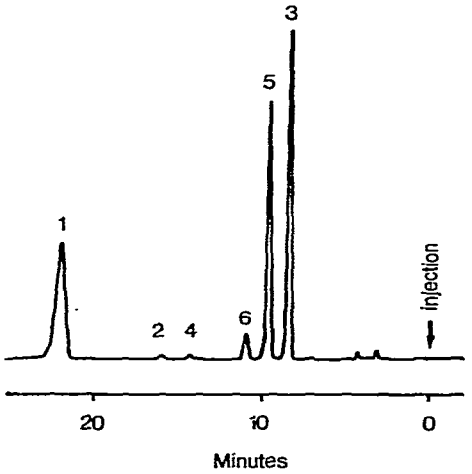


Fig. 3. HPLC separation of tetraethyluric acid. Column, μPorasil; solvent, *n*-heptane–isopropanol (80:1, v/v); flow-rate, 1 ml/min; detection, UV, 254 nm.

Preparative separation by adsorption chromatography of the ethylation product from pure uric acid revealed a complex mixture of compounds (Fig. 4), and those which could be identified as isomers of TEUA are numbered.

The separation of the ethylation reaction products by gas chromatography gave two major peaks (A and B in Fig. 5), similar to that reported previously[7]. When the six TEUA isomers, each isolated by reversed-phase HPLC, were gas chromatographed separately (Table I), it was found that TEUA 3 and 5 had identical retention

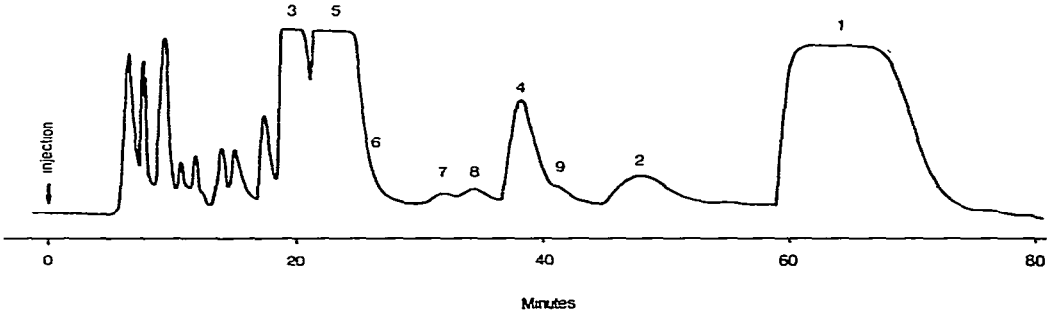


Fig. 4. Preparative HPLC separation of ethylation products. Column, Partisil 10 Magnum 9; solvent, *n*-heptane–isopropanol (40:1, v/v); flow-rate, programmed; detection, UV, 240 nm.

times, while that of TEUA 6 was only slightly shorter. These three isomers chromatographed as peak A and TEUA 1 as peak B (Fig. 5). TEUA 2 and 4 evidently decomposed on the column.

Calibration graphs constructed by using TEUA 3 and 5 were almost identical (Table II), and either of these could in fact be used for the determination of the corresponding isomer obtained from uric acid in serum. Additional results substantiating this estimate were obtained from similar measurements on TEUA 1, but it

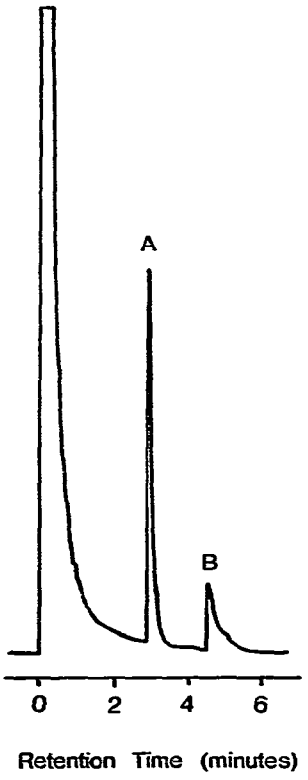


Fig. 5. Gas chromatography of TEUA isomers. Column, 3% OV-1

TABLE I

COMPARISON OF THE SEPARATION OF TEUA ISOMERS BY HPLC AND GLC

Reversed-phase HPLC peak No.	*GC retention time (min)*	*Corresponding GC peak*
1	6.3	B
2	Decomposes	
3	4.1	A
4	Decomposes	
5	4.1	A
6	4.0	A

TABLE II

CONSTANTS FOR CALIBRATION GRAPHS ($y = mx + c$) FOR TEUA 3 AND TEUA 5

Isomer	*m*	*c*
TEUA 3	0.381	0.053
TEUA 5	0.382	0.045

was found that some samples were contaminated, probably with the placticizer dioctyl phthalate, and these results therefore were not used.

The Reference Serum was analysed in triplicate and a set of results was obtained for each of the isomers TEUA 3 and 5. From the total of six separate estimates, a mean concentration ($\pm$ standard deviation) was calculated to be 4.31 $\pm$ 0.01 mg/dl (coefficient of variation 0.28%).

DISCUSSION

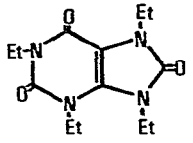
Stable-isotope dilution-mass spectrometry has recently been applied to the assay of organic molecules of clinical importance in serum[9]. The lack of adequate specificity, and hence accuracy, of routine uric acid assays has led us to investigate isotope dilution–mass spectrometry as a basis for a definitive method for measuring this analyte. Ideally, in such a method uric acid should be isolated in a pure form prior to its introduction into the mass spectrometer. However, the difficulty of extracting uric acid from serum with organic solvents and its low volatility for GC–MS analysis has made it necessary to adopt chromatographic and derivatization procedures.

The recovery of uric acid from pooled human serum by anion-exchange chromatography was 92 $\pm$ 2%. This was adequate for the present method as the use of stable-isotope-labelled uric acid as internal standard eliminates the need for quantitative recovery, on the assumption that the relative amounts of natural and labelled uric acid which are lost during the work-up are constant. This assumption would, of course, have to be thoroughly tested in elaborating this procedure for the purposes of a definitive method.

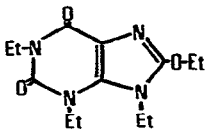
There are many methods for the alkylation of uric acid[10] and it is probable that they all give a complex mixture of products. For example, methylation of uric acid using diazomethane[11] was reported to give six tetramethyluric acid isomers. The present method was chosen because it allows direct ethylation of the column eluate.

It was reported[7] that ethylation of uric acid with diethyl sulphate gave two principal products, the purities of which were monitored by gas chromatography using a high-resolution capillary column. HPLC, however, showed that the same reaction gave a complex mixture of products (Figs. 1 and 4), some of which gas chromatographed as a single peak, with others decomposing on the column. Evidently, gas chromatography was unable to resolve the isomers and other unidentified products. Hence it was inappropriate to proceed with a GC–MS analysis before purifying the reaction mixture to permit ratio measurements on a single isomer. HPLC proved a suitable means of isolating the isomers, with adsorption chromatography being preferred because no further extraction is required and the eluent can be easily removed by evaporation.

There are 12 possible isomers of tetraethyluric acid. The likely structures of the three main TEUA isomers which are formed in the ethylation reaction[7] are shown in Fig. 6. From the HPLC behaviour, it is probable that TEUA 1, which chromatographed as the first peak on reversed-phase chromatography and as the last peak on adsorption chromatography, is 1,3,7,9-tetraethyluric acid, as this isomer contains three keto groups and is therefore the most polar. As this isomer gas chromatographs as peak B (Fig. 5), the structure suggested by its HPLC performance is at variance with that reported[7]. It is now known that peak A, which was previously assigned[7] the structure of 1,3,7,9-tetraethyluric acid on the basis of NMR data, is a mixture of isomers. Assignment of absolute structures by proton magnetic resonance spectroscopy was attempted in the present study, but was not possible because the three major isomers were found to have very similar spectra.



TEUA 1



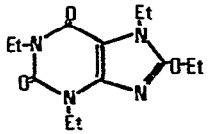


Fig. 6. Structures of three main TEUA isomers formed in the ethylation reaction.

The acceptance of the accuracy of stable-isotope dilution assays is based on the assumption that different isotopic forms of an element or compound behave identically. However, it is well known that the rates of reaction of heavy and light isotopes may differ[12]. For this reason, the reaction conditions were carefully controlled so that standard solutions and serum specimens were subjected to the same conditions; any isotope effect should be identical in sera and standards so as not to be a source of error. The proportions of the six TEUA isomers were found to change with reaction conditions such as temperature, pH and time.

A similar approach[13,14] to the accurate assay of uric acid in serum using a different ethylation procedure has demonstrated the presence of a small product

isotope effect, *i.e.*, a small difference (0.5%) was found between the isotope ratios in two different isomers. However, any difference in product isotope effect between TEUA 3 and TEUA 5 was smaller than the imprecision in the measurement of the isotope ratios.

The good agreement between the standard graphs for TEUA 3 and TEUA 5 strengthens confidence in the results obtained for the assay of the Tentative Reference Serum.

CONCLUSION

The procedure presented is a specific, accurate and precise method for the determination of uric acid in serum. The preliminary purification of uric acid by anion-exchange chromatography and the final separation of the TEUA isomers by HPLC ensure that a pure compound is being analysed. This specificity is enhanced by mass spectrometric measurement using selective ion monitoring.

The accuracy of the method is assured by inclusion of [1,3,9-^{15}N]uric acid as an internal standard so that quantitative isolation of uric acid and its derivatives is not required.

A value for the uric acid concentration (4.31 $\pm$ 0.01 mg/dl) has been assigned to the Tentative Reference Serum.

ACKNOWLEDGEMENTS

We thank Mr. A. C. S. Thomas for his assistance in applying the computer methods for acquiring and processing of ion ratios and Dr. F. L. Mitchell and Dr. S. S. Brown for helpful discussions and encouragement.

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