Determination of chelated iron in the urine

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SYNOPSIS A simplified wet-ashing technique is described for the determination of chelated iron in the urine, with special reference to DTPA-bound iron. The procedure dispenses with the need for quantitative transfer, and gives corresponding economy in time, labour, and glassware without loss of precision. The standard error of the mean estimate of a triplicate determination, using four reference standards, is $\pm 4 \mu\text{g}/100 \text{ ml}$; precision may be further increased by the use of a calibration curve based on many standards.

The determination of urinary iron has acquired new interest and importance with the increasing use of chelating agents in diagnosis and treatment. The estimation continues to have a reputation for difficulty and unreliability which, with the improved reagents now available, should not apply to the easily measurable iron concentrations that chelating agents produce in the urine. It has been found, however, that the presence of chelating agents interferes with the direct colorimetric estimation of that metal and non-ash methods have proved unreliable for the determination of DTPA-bound iron (Barry, 1967).

For the estimation of chelated iron in the urine it has been possible to simplify the traditional wet-ashing techniques without loss of accuracy. A method is described in which a small volume of urine is digested, and the iron content of the residue determined, without need for quantitative transfer; the only glassware required is Kjeldahl flasks and pipettes, and there is an overall economy of time and labour.

METHOD

PRINCIPLE A standard quantity of acid is used to ash 10-20 ml of urine to yield a dry residue which is re-dissolved in water in the same flask. The ferric iron in an aliquot is reduced with thioglycollic acid, and a pink colour is developed with bathophenanthroline sulphonate in the presence of an acetic acid-acetate buffer at pH 4.5.

PREPARATION OF GLASSWARE Glassware, containers, and boiling chips are washed in Pyronex (Diversey (U.K.) Ltd., Barnet, Herts.) detergent solution and tap water, soaked in 6N HCl and rinsed thoroughly in tap water, glass-distilled water, and ion-free water, and then oven-

dried. Boiling chips, supplied as Bauxilite white abrasive chips (The Universal Grinding Wheel Co. Ltd., London, S.W.1), have been shown to be rendered iron free by this treatment.

REAGENTS Analytical grade chemicals and glass-distilled water passed twice through a Deminrolit Mark 7 de-ionizing column have been used.

Bathophenanthroline sulphonate 4,7-Diphenyl-1-10-phenanthroline disodium disulphonate (Sigma Chemical Co.) 100 mg, is dissolved in 100 ml of water.

Acetic acid-acetate reagent Glacial acetic acid, 40 ml, is added to 100 ml. of aqueous 60% w/v sodium acetate ($\text{NaC}_2\text{H}_3\text{O}_2 \cdot 3\text{H}_2\text{O}$).

Thioglycollic acid 80% in water

Sulphuric acid-nitric acid mixture 1:1 v/v mixture of concentrated acids

Perchloric acid (HClO_4) 60%

Hydrogen peroxide (H_2O_2) 30% w/v

Stock standard iron solution (10 mg/100 ml) Ferrous ammonium sulphate hexahydrate (MW 392), 0.702 g, is transferred to a 1,000 ml volumetric flask and dissolved in about 800 ml of water, 0.5 ml concentrated sulphuric acid is added, and the solution is made up to the mark with water.

AMOUNT OF URINE ASHED The concentration of organic matter in a urine determines the amount that can be ashed by the standard 1.5 ml of acid; this can usually be judged from the total urine volume. It has been found that aliquots of 10 ml, 15 ml, and 20 ml are appropriate for six-hour urine volumes of less than 400 ml, 400 to 800 ml, and greater than 800 ml respectively. The iron yield from such aliquots permits accurate determination of the DTPA-provoked iron excretion in normal subjects. When it is known that the iron excretion will be very high, half-volumes of acid and 5 ml urine may be used. Heavy glycosuria results in much charring and reduces the volume of urine that can be easily digested.

ASHING PROCEDURE Urine, 10 ml, is pipetted into a 50-ml Kjeldahl flask containing 3 boiling chips; 0.5 ml of the $\text{H}_2\text{SO}_4\text{-HNO}_3$ mixture, and 1 ml of HClO_4 are added. The contents of the flask are brought quickly to the boil, and then allowed to boil gently on a digestion rack until a clear colourless residue is obtained. After it has cooled the residue consists of white crystals and a variable quantity of unused acid; according to the amount of acid remaining, which depends on the organic content of the urine, a further aliquot of up to 10 ml of urine may be added to the flask and digestion continued. The addition of the urine to the acid in two increments in this way has enabled the volume of urine that will be ashed by 1.5 ml of acid to be judged more exactly; it has also entailed heating a smaller quantity of fluid at any one time and eliminated the incidence of bumping. The final yield should be a white crystalline residue which appears not more than slightly moist; to obtain this a few drops of H_2O_2 may have to be added during the terminal stages of digestion.

At this stage the dry residue may be stored after sealing the mouth of the flask with Parafilm (A. Gallenkamp & Co. Ltd., London, E.C.2). Immediately before iron estimation 15 ml of water is pipetted into the flask; the contents are brought rapidly to near boiling and then allowed to cool. While the flask is still lukewarm it is inverted several times after its mouth has been tightly sealed with parafilm. An aliquot of the completely clear colourless solution is taken for iron estimation.

PREPARATION OF STANDARDS AND BLANKS Working standards have been routinely prepared in fresh, normal, uncontaminated urine, since urine digests more smoothly than water. It has been convenient to prepare a single working standard iron solution and to take 10 ml quadruplicates for ashing. Standard iron concentrations of 100 or 200 $\mu\text{g}/100\text{ ml}$ have been appropriate. Blanks are prepared for the urine from which the standard was made by ashing 10 ml quadruplicates; these are subsequently termed the standard urine blanks.

The H_2SO_4 , HNO_3 , and HClO_4 make negligible contributions to the background iron contamination. To correct for random contamination of the unknowns from the other reagents and glassware, triplicate reagent blanks are prepared by heating half-volumes of the acids for one hour, adding a few drops of H_2O_2 near the end; these residues are subsequently treated as the others.

BATHOPHENANTHROLINE SULPHONATE METHOD FOR IRON ESTIMATION The method of Goodwin and Murphy (1966) has been modified. Three ml of the unknown solution is pipetted into a disposable iron-free plastic tube (The Metal Box Co. Ltd., Plastics Division, London, W.1) and mixed with 3 drops of thioglycolic acid and 2.5 ml acetic acid-acetate reagent. The tube is stood for five minutes, then 0.4 ml bathophenanthroline is added and the contents mixed. Full colour development occurs after incubation for 30 minutes in a water bath at 37°C . The extinctions at $535\text{m}\mu$ are determined, setting the spectrophotometer to zero with distilled water. It has been convenient to use a Unicam SP600 and 2 cm glass cells; 1 cm cells may be used, particularly when very

high urinary iron concentrations are being routinely determined. When the reading exceeds $E_{535}^{2\text{cm}} 1.00$ the solution is reestimated after appropriate dilution; blanks should be prepared in similar dilution although it has been found that this does not significantly reduce the blank extinction value.

The extinctions of the standard quadruplicates are corrected by subtracting the mean extinction of the standard urine blanks (usually about 0.090). The extinctions of the unknowns are corrected by subtracting the mean of the three reagent blanks (usually about 0.070). The iron concentrations of an unknown urine is given by

$$\frac{E_{535}^{\text{unknown}}}{E_{535}^{\text{standard}}} \times \text{iron concentration of standard} \times \frac{V_s}{V_u}$$

where V_s (ml) is the aliquot of standard ashed, and V_u (ml) the aliquot of unknown urine ashed.

RECOVERY EXPERIMENTS

IRON RECOVERY FROM ASHED AND NON-ASHED STANDARDS Working standards of iron in urine, 100 $\mu\text{g}/100\text{ ml}$ and 40 $\mu\text{g}/100\text{ ml}$, were prepared. Three ml duplicates were estimated for iron directly without prior ashing. Ten ml duplicates of the same standards were ashed, and 3 ml aliquots of the residues estimated for iron. The extinctions, after appropriate blank subtraction, for the ashed and non-ashed duplicates are shown in Table I; the percentage recovery of the ashed duplicates takes into account the 1.5 times dilution of the iron in the residues. Mean iron recovery by the ashing technique is 100.5%.

TABLE I

COMPARISON OF THE RECOVERY OF NON-CHELATED IRON ADDED TO URINE USING NON-ASH AND WET-ASH METHODS

Iron Concentration ($\mu\text{g}/100\text{ ml}$)	$E_{535}^{2\text{cm}}$ Non- ashed Urine	$E_{535}^{2\text{cm}}$ Ashed Urine	Percentage Recovery after Ashing ¹
40	0.162	0.113	104
	0.172	0.115	100
	0.424	0.285	101
100	0.424	0.273	97

¹Percentage recovery after ashing takes into account the 10 in 15 dilution of iron involved in the method.

RECOVERY OF CHELATE-BOUND URINARY IRON The DTPA in an iron-containing solution must be completely destroyed to render the iron completely available for colour formation above pH 2. The extinctions for many standard iron replicates are shown in Table II. Iron recovery after ashing is not affected by the addition of DTPA or desferrioxamine to the working standards in concentrations of 250 mg/100 ml.

REPRODUCIBILITY AND PRECISION The reproducibility of the method is indicated by the small standard errors of the means of the extinction values for the standard iron solutions in Table II. Figure 1 indicates that over the range 40-200 $\mu\text{g}/100\text{ ml}$ there is a linear relationship

TABLE II

RECOVERY OF CHELATE-BOUND IRON

Iron Concentration ($\mu\text{g}/100\text{ ml}$)	Number of Replicates	$E_{535}^{2\text{cm}}$ mean $\pm$ S.E.M.
40	5	0.110 ± 0.002
40 + DTPA	6	0.108 ± 0.003
80 + DTPA	4	0.217 ± 0.007
100	16	0.255 ± 0.005
100 + DTPA	7	0.254 ± 0.007
100 + desferrioxamine	8	0.256 ± 0.006

$E_{535}^{2\text{cm}}$ for standard iron replicates in urine after ashing. DTPA or desferrioxamine was added to those standards indicated in concentration 250 $\text{mg}/100\text{ ml}$.

between iron concentration and extinction. Analysis of variance reveals no significant deviation from linearity, a residual error of $E_{535}^{2\text{cm}}$ 0.01375 and a regression line slope of $E_{535}^{2\text{cm}}$ 0.00263/ μg iron/100 ml. The standard error of the mean estimate of the iron concentration in an unknown sample may be expressed in terms of the residual error (s), the slope (b), and the number of replicates in the unknown (Nu) and standard (Ns) groups. For this type of data the relationship approximates to

$$\text{SEM} = \frac{s}{b} \sqrt{\frac{1}{N_s} + \frac{1}{N_u}} \quad (\text{Finney, 1964})$$

and it may be calculated that, with four standard and three unknown replicates, the SE of the mean estimate is $\pm 4.0\text{ }\mu\text{g}/100\text{ ml}$.

DISCUSSION

The main purpose of the ashing method described has been to destroy the chelating agent and render all the iron available for colour formation. Since the method was specifically developed for the determination of DTPA-bound urinary iron in diseases not necessarily complicated by iron overload it allows for the inorganic solute concentration of a dilute urine to be increased by up to 33% in the residue. The use of bathophenanthroline (molar extinction coefficient of ferrous complex 22,400) and 2 cm cells has conferred sufficient sensitivity to permit the accurate measurement of the 60-300 μg of iron excreted in six hours by normal subjects after the intravenous administration of DTPA, 16.8 mg/kg . The method has been equally applicable to desferrioxamine-bound iron. For the routine determination of iron excretion in subjects with increased chelatable iron it may be found more convenient to use half-volumes of acid and urine and 1 cm cells. It must be emphasized that the method is not suitable for the accurate determination

FIG. 1. Relation between $E_{535}^{2\text{cm}}$ and iron concentration in 40 replicates taken from 14 separately prepared standards representing 4 different concentrations of iron.

of physiological iron excretion, where the urinary iron concentration approximates to the SE of the estimate given by the method; it may, however, be used to define the upper limit of the baseline iron excretion.

The elimination of the need for quantitative transfer and the use of disposable plastic tubes where indicated confer considerable saving in glassware and labour. Data have been presented to show that these advantages are not accompanied by lack of precision.

This method has been developed from that of Dr. L. W. Powell (1966) and his colleagues, to whom I owe much help. Thanks are due to Professor D. N. Baron, Dr. Barbara Billing and Dr. V.M. Rosenoer for encouragement, advice, and criticism. The work was performed while the author was in receipt of a Saltwell Fellowship of the Royal College of Physicians.

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