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A cost-effective modified micromethod for measuring urine iodine

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Abstract. A modified micromethod for measuring urine iodine was successfully established and validated. The micromethod showed good correlation with the method used by several World Health Organization (WHO) collaborative laboratories (y=0.9342x + 4.6213; r=0.962; p=0.01; n=50). The micromethod also showed good agreement when compared to the reference WHO method. The sensitivity of the assay was 13.809ug/L (n=8) and mean recoveries were 114, 103 and 106% at concentrations of 30, 40 and 50ug/L (n= 3) respectively. At iodine concentrations of 51 ± 15.5, 108 ± 32.4 and 149 ± 38.6ug/L, intra-assay coefficient of variations (CVs) were 13%, 7% and 5% respectively (n=20), and inter-assay CVs were 10%, 15% and 7% respectively (n=10). The assay showed good linearity plot (y=1.0407x + 60.451; r=0.993; n=3).

INTRODUCTION

In Malaysia, efforts have been made by the Ministry of Health (MOH) towards elimination of Iodine Deficiency Disorders (IDD) in the country since there are still a few remote areas which have iodine deficiency as a problem, especially in Sarawak where prevalence of endemic goitre ranged from 20% to over 90% (Kiyu & Zainab, 1993), as observed from studies (Chen & Lim, 1982; Yap, 1985; Chen & Yap, 1988) done in 1980's. To date, along with the implementation of iodization of drinking water and legislation of Universal Salt Iodization in the IDD high prevalence areas, median urine iodine (mUI) as a sensitive biochemical indicator towards recent changes in dietary iodine intake has been used to monitor updates of population IDD status. As a reference laboratory, the responsibility of the Institute for Medical Research (IMR) is to provide technical advice to all iodine laboratories in MOH, supply field test kits for measuring iodine in salt and water to all State Health Departments in the IDD high prevalence areas and carry out evaluation study to determine effectiveness of the program.

A few methods have been developed to determine urinary iodine concentration, beginning with the earliest one developed by Sandell & Kolthoff (1937). Later, modifications to the method were reported. The colorimetric method by Dunn *et al.* (1993) was based on the Sandell-Kolthoff reaction where urine samples were predigested with chloric acid, followed by Pino *et al.* (1996) who further improved the method by replacing chloric acid with ammonium persulfate. Compared to chloric acid, ammonium persulfate is nonhazardous and nonexplosive. In the micromethod developed by Ohashi *et al.* (2000), the use of multichannel micropipette to add arsenious acid and ceric ammonium sulfate solutions into microtitre plate has enabled more samples to be analysed. At the moment however, all iodine laboratories in the country (including IMR) are using the conventional chloric acid method which is not only hazardous but also laborious and technically demanding, and only allows

analysis of 42 samples per run. This study therefore described an adaptation of the micromethod by Ohashi *et al*. (2000), modified such that it can be easily performed in laboratories without the need to purchase expensive specialised equipment.

MATERIALS AND METHODS

MATERIALS

Chemicals

Ammonium persulfate and arsenic trioxide were obtained from MERCK (Darmstadt, Germany) and SIGMA-ALDRICH (St. Louise, MO, USA), respectively. Perchloric acid (70%), potassium chlorate, sodium chloride, sulphuric acid, ceric ammonium sulfate and potassium iodate were obtained from BDH Laboratory Supplies (Poole, England). Sodium hydroxide was obtained from May & Baker Ltd. (Dagenham, England).

Standards

Standards were prepared through serial dilution of the stock potassium iodate (500 µg/L) solution yielding final concentrations of 10, 40, 80, 120, 200 µg/L, while deionized water was used as 0 µg/L standard (Table 1).

Samples

Urine samples used to prepare pooled controls and for precision, recovery, linearity and method comparison studies were obtained from the staff of the Cardiovascular, Diabetes and Nutrition Research Centre, IMR, and leftover samples from several studies done earlier.

Instruments

For absorbance reading in WHO reference method, a Metertek SP-850 spectrophotometer was used at 420 nm wavelength. Absorbance in the modified micromethod was measured using an Anthos 2010 microplate reader (Anthos Labtec Instruments, Austria) at 405 nm wavelength. For dispensing reagents into the 96-well microtitre plates (MTP), Nichiryo model 8800 repeating multichannel micropipette was used. Shaking of microtitre plates during incubation was done at 600rpm using Labnet Orbit P4 (3mm orbit) orbital shaker (Labnet International Inc.).

METHODS

For both WHO reference method and modified micromethod, urinary iodine was estimated based on Sandell-Kolthoff reaction as stated below :

$$As^{3+} + I_2 \longrightarrow As^{5+} + 2I^-$$

$$2Ce^{4+} + 2I^- \longrightarrow 2Ce^{3+} + I_2$$

The yellow colored ceric ion (Ce^{4+}) is reduced to colorless cerous ion (Ce^{3+}) by iodide ion (I^-). The time taken for the color disappearance is inversely proportional to the amount of iodide catalyzing it.

Each assay consisted of a set of 6 standards and two sets of low, medium and

Table 1. Schematic diagram showing the preparation of standard solutions

Standards	Volume added		Final concentration (µg/L)
	Standard solution	Deionized water (µl)	
S6	1000 µl of WS*	1500	200
S5	720 µl of S6	480	120
S4	800 µl of S5	400	80
S3	600 µl of S4	600	40
S2	200 µl of S3	600	10
S1	0 µl	600	0

WS* = Working standard : 0.5 µg/ml = 500 µg/L potassium iodate.

high quality control samples placed at beginning and end of assay.

WHO Reference Method (WHO, 2001)

Solutions

Digestive agent: 114.1 g ammonium persulfate was dissolved in deionized water to a final volume of 500 ml.

Arsenious acid solution : 5 g arsenic trioxide and 12.5 g NaCl in 250 ml deionized water and 100 ml 5 N sulphuric acid were gently heated until dissolved. Deionized water was then added to a final volume of 500 ml.

Ceric ammonium sulfate : 2.4 g ceric ammonium sulfate was dissolved in 3.5 N sulphuric acid to a final volume of 50 ml.

Experimental procedure

250 µl of urine samples, standards and controls were added into respective 16 x 124 mm glass test tubes, followed by the addition of 1ml ammonium persulfate. Mixtures were mixed and heated at 100°C for 1 hour. Tubes were cooled to ambient temperature (AT) before addition of 2.5 ml arsenious acid solution, followed by further incubation at AT for 15 minutes. Ceric ammonium sulfate (300 µl) was then added to each tube at 30 second-intervals. After another 30 minutes incubation at AT, absorbance was read at 420 nm. Absorbance of subsequent reaction mixtures was read at intervals such that the reaction time was 30 minutes after addition of ceric ammonium sulfate. Normally, a maximum of 60 tubes per assay could be run at a time. The iodine concentrations of the unknown urine samples were determined from the standard curve of absorbance versus iodine concentration (µg/L).

Modified micromethod

This is a modified method, a combination of WHO (2001) reference method and method by Ohashi *et al.* (2000).

Solutions

Digestive agent : 114.1 g ammonium persulfate was dissolved in water to a final volume of 500 ml (WHO, 2001).

Arsenious acid solution : 3.5 g NaOH and 5 g arsenic trioxide were heated until dissolved in 100ml deionized water. Solution was then cooled to RT, placed in a water bath and 16 ml 18 M sulphuric acid was added slowly. 12.5 g NaCl was added and stir until dissolved. Deionized water was added to a final volume of 500 ml (Ohashi, 2000).

Ceric ammonium sulfate : 2.4 g ceric ammonium sulfate was dissolved in 3.5 N sulphuric acid to a final volume of 50 ml. Prior to assay, 3 ml of solution was diluted with 9 ml 3.5 N sulphuric acid.

Experimental procedure

250 µl of urine samples, standards and controls were added into respective 16 x 124 mm glass test tubes, followed by the addition of 1ml ammonium persulfate, mixing and heating at 100°C for 1 hour. After the tubes were cooled to AT, 30 µl of the digested samples / standards / controls were transferred into corresponding wells of a 96-well flat-bottomed polystyrene MTP. This was followed by addition of 60 µl of arsenious acid solution and 30 µl 0.019 M ceric ammonium sulfate into each well using repeating multichannel micropipette. The MTP was tapped gently to mix the reaction mixtures and followed by incubation at AT on a microplate shaker at 600rpm shaking speed. Absorbance was read at 405 nm using a microplate reader. The iodine concentrations of the unknown urine samples were determined from the standard curve of absorbance versus iodine concentration (µg/L).

Method Validation

Assay Sensitivity

Eight blanks were run in an assay and sensitivity was determined from the standard curve at 2 standard deviation (SD) from mean absorbance of the blanks. The average sensitivity from 4 assays was then calculated to obtain the assay sensitivity.

Assay Precision

Three pooled urine controls with iodine concentrations of 51 ± 15.5, 108 ± 32.4 and

149 ± 38.6 µg/L were used in determining assay precision. The intra-assay imprecision was evaluated using n=20 replication of controls in an assay and average coefficient of variation (CV) was calculated from 10 assays. The same set of data was then used to determine the inter-assay imprecision.

Assay Recovery

Potassium iodate solutions at 30, 40 and 50 µg/L were used to spike pooled urine samples with concentrations at 51 ± 15.5, 65 ± 7.2 and 108 ± 32.4 µg/L. Pooled and spiked urine samples were run in triplicates in 3 separate assays. Recovery was calculated using the formula (Sullivan et al., 2000) :

$$\frac{([\text{Spiked urine}] - [\text{Pooled urine alone}])}{[\text{Concentration of iodine added to urine specimen}]} \times 100\%$$

Assay Linearity

Five levels of urine pools were prepared : 0, 25, 50, 75 and 100% dilution (Westgard, 2003) of Pool 1 (51 ± 15.5 µg/L) and Pool 5 (149 ± 38.6 µg/L). Each pool was run in triplicates in 3 separate assays and mean of measured values were used to draw the 'Linearity Plot' (Westgard, 2003).

Method comparison

Fifty urine samples were analyzed using modified micromethod and WHO reference method in duplicates in 3 separate assays. Results were used to draw the 'Difference Plot' and 'Comparison Plot' to see the relationship between the methods (Westgard, 2003).

Calculations

Standard curve plotting and unknown urine samples concentration determination were obtained using MULTICALC software from Perkin Elmer. Correlation coefficient, r, in the 'Comparison Plot' was determined with Pearson's Correlation using Statistical Package for Social Sciences (SPSS) version 11.5. Results for sensitivity, precision, recovery, linearity and 'Difference Plot' were determined using Microsoft Excel 2003 spreadsheet software.

RESULTS AND DISCUSSION

In this study, we modified and validated a micromethod for measuring iodine in urine using laboratory equipments readily available in the IDD Laboratory in IMR with minimal cost for reagents. The modified method was designed as such that it could measure iodine concentration between 0 – 200 µg/L (Figure 1).

From the method validation results, it was found that modified micromethod was comparable to WHO reference method (Sullivan *et al.*, 2000). Sensitivity of the assay, defined as the lowest detectable concentration of urine iodine was 13.809 µg/L (n=8) compared to <10 µg/L(n=8) obtained for the WHO reference method. Assay imprecision gave fairly good results. The intra-assay coefficient of variations (CVs) at iodine concentrations of 51 ± 15.5, 108 ± 32.4 and 149 ± 38.6 µg/L were 13, 7 and 5% (n=20) respectively, whilst the corresponding inter-assay CVs were 10, 15 and 7% (n=10) respectively. The WHO reference method gave both intra- and inter-assay imprecisions of <10% (n=5). Good analytical recoveries of 114, 103 and 106% were obtained for iodine concentrations of 30, 40 and 50 ug/L respectively, comparable to that obtained using WHO reference method (98.5%; n=3). Assay linearity is presented in Figure 2, depicting a good 'Linearity Plot' (y = 1.0407x + 60.451, r = 0.993, n=3).

Results for method comparison between modified micromethod and WHO reference method is shown in Figure 3. Both methods correlated well (y=0.9342x + 4.6213; r=0.962; p=0.01; n=50) with mean difference (d) of 2.012 ug/L and SD of 11.137 ug/L for urinary iodine concentrations of 24 - 190 ug/L. Based on 'Difference Plot', the modified micromethod showed good agreement with WHO reference method (Figure 4). Most of the measurement differences between the methods lies within ± 2SD.

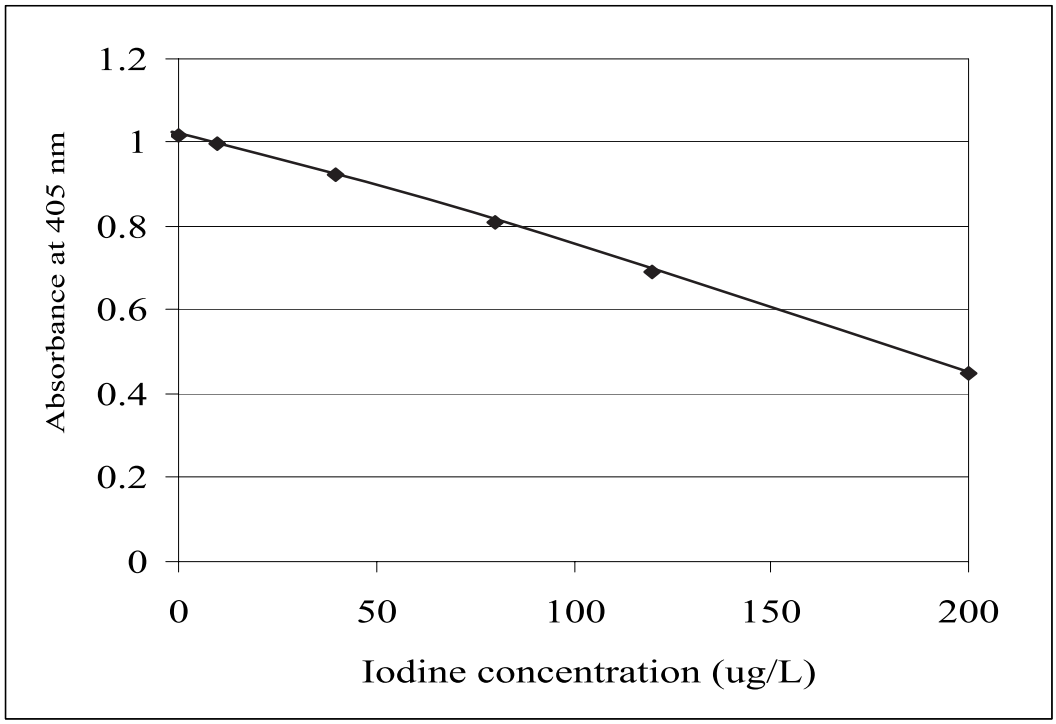

Figure 1. Standard curve for iodine measurement using modified micromethod.

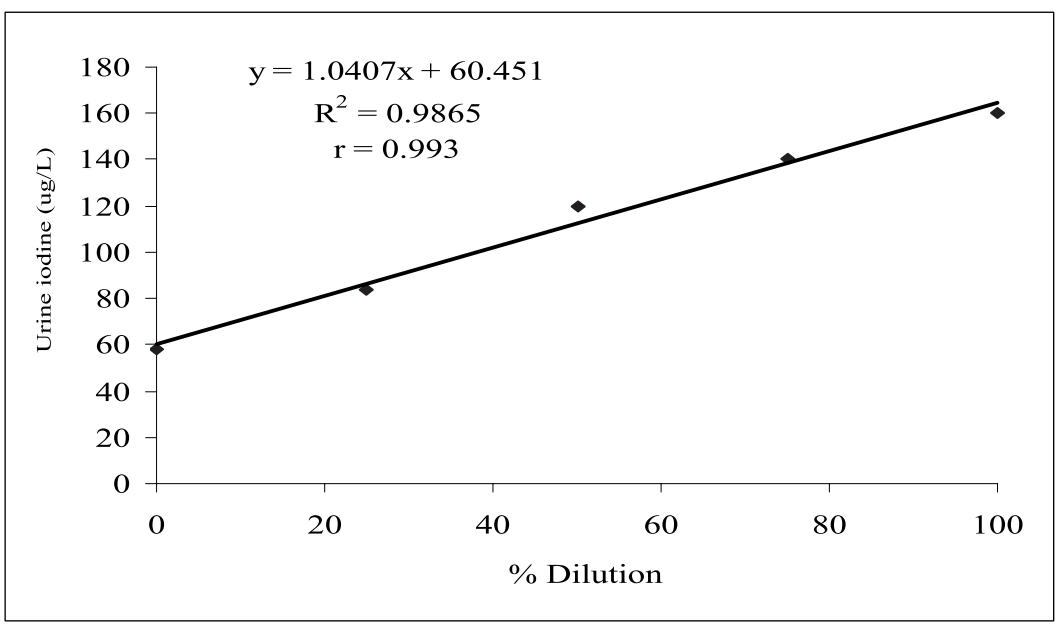

Figure 2. Linearity Plot for urine iodine measurement using modified micromethod.

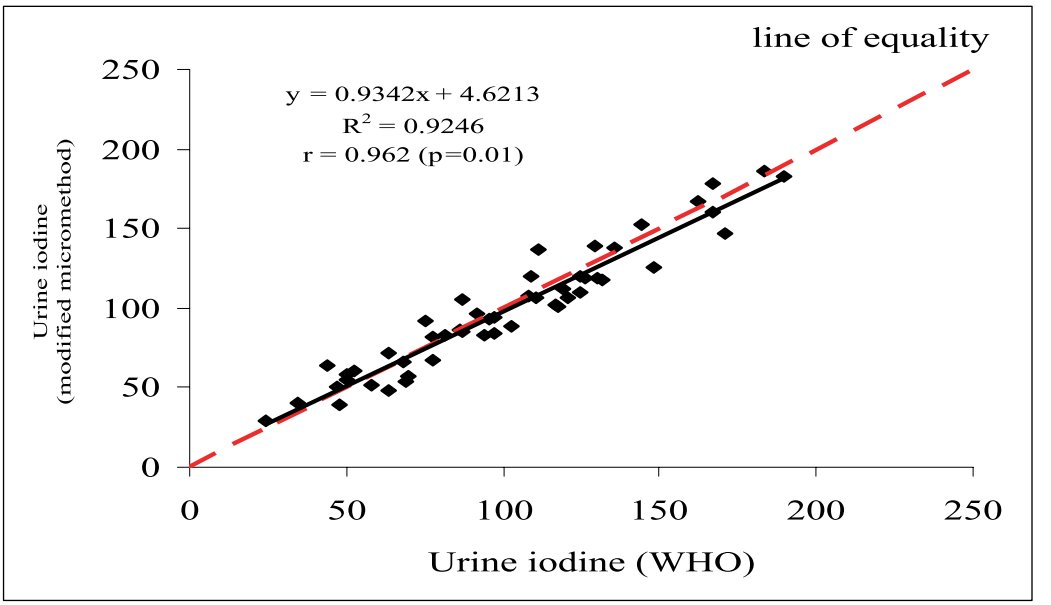

Figure 3. Correlation between urine iodine measured using modified micromethod vs. WHO reference method.

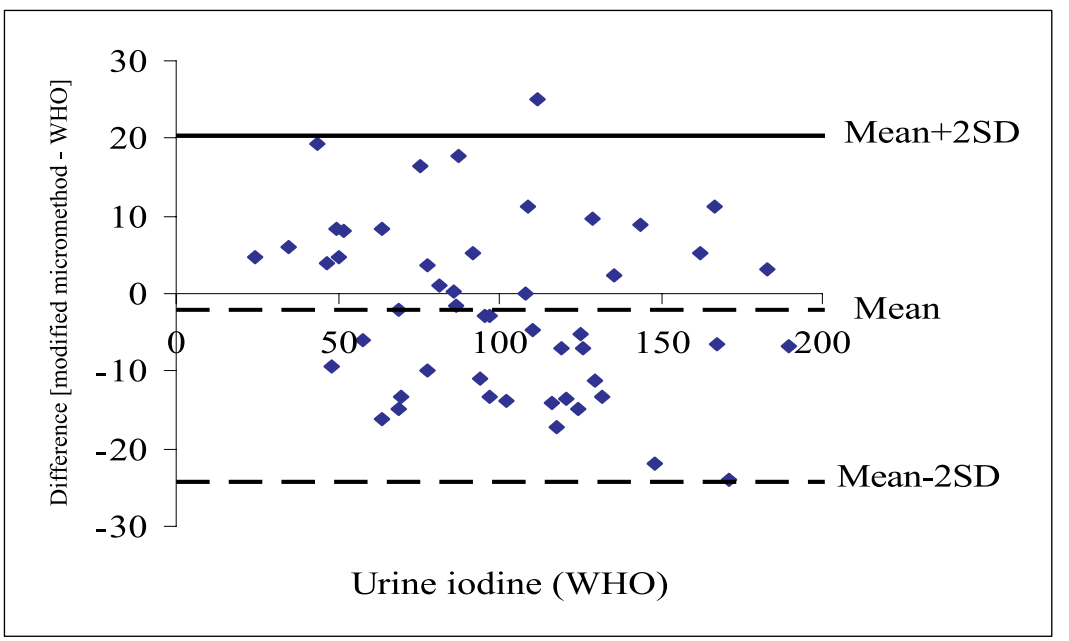

Figure 4. Difference Plot for urine iodine measurement using modified micromethod compared to WHO reference method.

In the micromethod described by Ohashi *et al.* (2000), urine digestion had to be carried out in a heat-resistant but more costly polypropylene MTP, and besides the need to use a special cassette sealer to prevent evaporation of samples, an oven is

also required for heating. The method also required the tedious process of transferring the samples into another MTP before reading the absorbance in a microplate reader. In contrast, our micromethod continued to use glass test tubes and heating block for digestion, thereby avoiding the need to purchase new instruments. Unlike the methods by Dunn *et al*. (1993) and WHO (2001) which used glass test tubes throughout the assay, we transferred the digested urine samples into a 96-well MTP and by using a repeating multichannel micropipette to add arsenious acid and ceric ammonium sulfate, and simultaneous absorbance reading of all 96 wells, we were able to analyse up to 78 samples in a single assay.

In conclusion, this study has successfully established a modified micromethod of urine iodine. The absorbance reading in 96-well MTP will allow more samples to be analysed at a time, thus, shortening the turnaround time. This method requires lesser amount of chemicals, thereby, saving cost of running IDD laboratories. Compared to the chloric acid method, the amount of chemical waste produced is less, and is definitely a safer method to use since ammonium persulfate is less hazardous. This simple method is therefore recommended for use by all iodine laboratories in the Ministry of Health Malaysia and any other IDD laboratories with heavy workload.

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