Measurement of Urinary Iodide Levels by Ion-Selective Electrode: Improved Sensitivity and Specificity by Chromatography on Anion-Exchange Resin

by Guy E. Abraham, MD, Jorge D. Flechas, MD
and John C. Hakala, RPh

Introduction

The last national nutritional survey (NHANES III 1988-1994) revealed that 15% of the US adult female population is iodine-deficient as defined by the World Health Organization: levels of iodine/iodide (I) below 50 µg/L.[1] That is one out of every seven female patients walking into a physician's office. Yet, rarely do physicians order urine I levels, even in patients with simple goiter and hypothyroidism. Such patients are usually prescribed thyroid hormones. There is convincing evidence that I deficiency predisposes to fibrocystic disease of the breast (FDB) and breast cancer.[2-8] Administration of thyroid hormones to I-deficient women increased further their risk of breast cancer.[9] Forty years ago, the risk ratio for breast cancer in our population was 1:20 and now it is 1:8,[10] coincident with an increased prevalence of I deficiency in our population.[1]

To encourage physicians to perform routine urine I determination in their practice, a simple method will be described to accurately measure urine I levels. This technique uses quantification of iodide by a potentiometric method, using an ion-selective electrode (ISE). Urinary iodide is measured by the electromotive force (EMF) generated on the iodide-selective electrode due to the presence of iodide in the urine sample. Within a certain range of iodide concentrations, there is a linear relationship between the logarithm of iodide concentration and the EMF generated.

The concentration of chloride in urine is usually 1 million-fold higher than iodide, and there is a significant interference by chloride in the analysis of urinary iodide by the ISE method. To prevent this interference, purification of the urine sample by anion exchange chromatography is performed prior to ISE measurement. This assay is simple and quick, with results within one hour, if the ISE measurement is performed in the physician's office. We also present preliminary data on an I-loading test to assess I sufficiency of the whole human body.

Motivating Factor in the Development of the Assay

Mainland Japanese women have a very low incidence and prevalence of FDB and breast cancer.[11] Several investigators have proposed that the essential element I was the protective factor in mainland Japanese.[2-8] If, indeed, the essential element I is the postulated protective factor, the administration of I to American women in amounts equivalent to that consumed by mainland Japanese women would be expected to protect them from breast cancer and improve FDB, as previously proposed by Stadel for breast cancer[12] and confirmed for FDB by Ghent, *et al*.[5] Based on data supplied by the Japanese Ministry of Health, the average daily I intake by mainland Japanese is 13.8 mg.[13]

We evaluated the effect of two drops of Lugol solution in tablet form containing 5 mg of iodine and 7.5 mg iodide as the potassium salt (Iodoral®, Optimox Corporation, Torrance, CA), administered daily for three months to 10 normal women. Informed consent was obtained from all subjects participating in the studies described in this manuscript. This supplement had no adverse effect on ultrasonometry of the thyroid gland, the serum levels of thyroid hormones, blood chemistry, hematology, and urine analysis.[13] This form and amount of I was chosen because it was widely prescribed during the early and mid 1900s for I replacement therapy.[13-16] The amount of 12.5 mg of I present in two drops of Lugol is very close to the estimated mean daily intake of 13.8 mg I by mainland Japanese.[13]

According to the medical literature, urinary I level is the most valid index of I intake.[17,18] Using a commercial laboratory (Doctor's Data Inc., Chicago, IL), we followed the pattern of 24-hour urinary I excretion before and after ingestion of one tablet of Iodoral® in two male and three female subjects. To our surprise, both male and female subjects who ingested one tablet of Iodoral® containing 12.5 mg I, excreted in their 24-hour urine samples only 10-30% of the I ingested, with a mean of 20% (Table 1). There are two possible explanations for this finding: low bioavailability of the solid dosage form of the I tablet or high retention by the body. If this was due to low bioavailability, prolonged administration of this I supplement should not result in an increased urinary excretion; otherwise, as the body becomes more I-sufficient, a greater percentage of the ingested I would be excreted. In order to elucidate the cause of this low I

Table 1

Urinary Excretion of Iodide in 5 Subjects on a Supplement Containing 12.5 mg of Iodine/Iodide (Iodoral®)

Subject	Sex	Age (years)	Height (inches)	Weight (pounds)	Urinary I Concentration (mg/24 hrs)		
					Pre	After 1 Day	After 30 Days
1	F	21	60	128	0.030	1.10	12.00
2	F	19	64	130	0.022	2.00	1.20
3	F	22	68	122	0.053	2.40	4.50
4	M	55	67	168	0.140	3.80	8.10
5	M	40	73	175	0.180	3.10	5.50
	$\overline{X}$	31	66	144.6	0.085	2.48	6.26
	SD	13	9.2	23	0.106	1.41	5.50

excretion, we continued the administration of I in those subjects for one month. Then, we repeated the 24-hour urine collection, and I was measured again in the collected urine samples.

Following one month of daily ingestion of one tablet of Iodoral®, the excretion of I increased to 36-96% of the oral amount in four of the subjects with a mean of 50% in the five subjects (Table I). Female Subject #2 excreted only 10% of the oral dose after one month of supplementation. She had the lowest baseline I level (0.022 mg/24 hr) and the greatest I retention after one and 30 days. The only distinctive feature of this subject was mammomegaly (size 40D). This would suggest a very important role of the mammary glands in the requirement for I by the whole human body. To our knowledge, the above findings have not been previously reported. The implication of such observation is that an I-loading test could be developed to assess not just thyroid sufficiency but I requirement of the whole human body. However, for such a test to be practical, one month duration is too long. So, the next alternative was to progressively increase the amount of I in a single dose and to measure urinary I excretion in order to find the amount of ingested I that would result in the greatest differences between individual urine I levels, as an index of degree of whole body I sufficiency. The standard deviation from the mean value could be used as an index of the between-individual variations.

Table 2

Urinary Excretion of Iodide in 6 Subjects Before and After Ingesting One, Two, and Three Tablets of a Supplement Containing 12.5 mg of Iodine/Iodide (Iodoral®)

Subject	Sex	Age (years)	Height (inches)	Weight (pounds)	Urinary I Concentration (mg/24 hrs)			
					Pre	1 Tablet	2 Tablets	3 Tablets
1	F	42	62	171	0.160	2.70	4.90	14.00
2	F	51	65	113	0.067	2.60	5.30	9.90
3	F	42	62	110	0.064	3.10	6.00	6.80
4	M	51	69	160	0.250	2.60	6.30	11.00
5	M	63	68	199	0.11	3.30	6.50	7.70
6	M	70	70	200	0.093	2.50	5.40	7.50
	$\overline{X}$	53	66	159	0.124	2.80	5.70	9.50
	SD	20	5.7	20.5	0.047	0.14	0.35	4.60

Another group of six subjects, three males and three females, was evaluated with 24-hour urinary I levels before and after ingesting one, two, and three tablets of the same preparation. The subjects retained approximately 80% of the I content of one and two tablets (Table 2). But with three tablets containing 37.5 mg of I, there was a two-fold difference in I excretion, with a range of 6.8-14 mg I (18-37%) (Table 2). The means ± SD of urinary I levels (mg/24 hr) for the three doses of I were: 2.8±0.14 (CV = 5%) for one tablet of Iodoral®; 5.4±0.35 (CV = 6.5%) for two tablets; and 9.48±4.6 (CV = 48.5%) for three tablets. There was a 10-fold increase in the coefficient of variation around the mean value at three tablets of the I supplement, compared to one and two tablets. Subject #3 who retained the most I, with only 6.8 mg (18%) in the 24-hour urine collection, suffered from severe FDB, again pointing to the mammary glands as an important organ of I utilization. These results suggest that the measurement of urinary I levels before and after administration of three tablets of Iodoral®, could be used as an I-loading test to assess I sufficiency of the whole human body.

For the I-loading test to be widely used in the physician's office, the measurement of urine I levels would be best performed *in situ*, using a simple method with non-hazardous reagents. The values for I presented in Tables 1 and 2 were obtained by a procedure called Induction Coupled Plasma-Mass Spectrometry (ICP-MS). The equipment required for this procedure is extremely expensive and very complex in its utilization. The ISE method is very simple, requiring only the following two reagents: water and sodium nitrate. Sodium nitrate is used in the ISE method as an Isotonic Strength Adjuster (ISA) at an initial concentration of 5 Moles/L to improve performance of the ISE electrode. The ISA is diluted one part ISA to two parts of iodide standards and urine samples. The final concentration of $NaNO_3$ in the assay is 1.66 Molar. The ISE procedure does not require any special precaution, beside the usual good laboratory practice. Water and $NaNO_3$ are used in the ISE measurement and in the chromatographic purification of urine samples on anion-exchange resin, simplifying the procedure.

Measurement of Iodide in Biological Fluids by Ion-Selective Electrode: A Review of Pertinent Publications

Urinary Iodide: In 1983, Cooper and Croxson[19] wrote a letter to the editor published in the *Journal of Clinical Chemistry*, describing their unsuccessful attempt to measure urine iodide levels in New Zealanders by the direct ISE method, using equipment from Thermo Orion (Beverly, MA). The high concentration of chloride pre-

sent in urine samples interfered in the assay. They were unable to achieve reliable and reproducible results. They postulated that chloride was the interfering substance due to the persistence of this interference following deiodization of urine by chromatography on anion-exchange resin. This procedure retained iodide on the column, but chloride was eluted in the chromatographed urine. Not only did the presence of chloride render the assay non-specific for iodide, but it also caused a decrease in sensitivity by one order of magnitude. With standards of iodide in water, the sensitivity was 10^{-6} M (0.127 mg/L), but with standards in deiodized urine, the sensitivity was 10^{-5} M (1.27 mg/L). It is surprising that these authors did not carry their experiments to the next logical step: use the same anion-exchange resin to purify iodide from chloride. Since chloride was eluted with the chromatographed urine, but iodide was retained on the column, they could have used a high ionic strength buffer to elute the iodide fraction afterward. The urinary I levels of New Zealanders are 10-20 times less than the sensitivity of 1.27 mg/L. These authors concluded that "on the basis of chloride error, the iodide-selective electrode is unsuited for the accurate experimental determination of iodide in urine."

In 1986, Yabu, *et al*[20] came to the realization that the New Zealander's problem was not a problem at all for mainland Japanese. Although they confirmed Cooper and Croxson's findings that the assay was not specific below 1.27 mg/L, mainland Japanese excreted higher levels of urinary iodide than 1.27 mg/L. In 163 urine specimens analyzed, only one specimen had a concentration below 1.27 mg/L. They observed I levels in these urine samples ranging from 0.6 mg/L to 17.4 mg/L. If those I levels are expressed as mg/24 hr and assuming an average 24-hour urine volume of 1.5 L, the range of I excretion per 24-hour would be 1-25 mg in these 163 Japanese subjects. This range is in agreement with the estimated average daily I intake of 13.8 mg I in mainland Japanese.[13]

The iodide levels observed in the mainland Japanese were two orders of magnitude higher than urinary iodide concentrations in New Zealanders — for that matter, in citizens of the Western World.[13] In 1993, Kono, *et al*,[21] using direct measurement of urine iodide in 2,956 men and 1,182 women, confirmed the reliability of the direct ion-selective electrode assay in urine samples from mainland Japanese subjects. However, Yabu and Kono were not able to measure accurately iodide levels below 1.27 mg/L due to chloride interference. Above that level, they achieve excellent correlation with an established assay for urinary iodide, the ceric ion-arsenious

acid method.[22] It is of interest to note that the electrode and meter used in the two publications from Japan were obtained from Radiometer (Copenhagen, Denmark). This company supplies a procedure to perform direct measurement of urinary iodide with its equipment.

Milk Iodide: In 1980, Lacroix and Wong measured iodide directly in cow's milk by ion selective electrode.[23] They reported a value ranging from 0.14-0.35 mg/L, when the milk analyzed was taken raw from individual cows. Market milk contained mean levels ranging from 0.52-0.70 mg/L. These authors did not perform experiments to prove the specificity of their procedure. In 1984, Gushurst, *et al,*[24] measured iodide in human milk by ion-selective electrode. They observed values ranging from 0.029-0.45 mg/L. Since the concentration of chloride in milk is 10 times less than in urine,[25] they were able to measure iodide levels down to 0.029 mg/L. The studies performed with milk samples used iodide-selective electrode and meter from Thermo Orion. The authors of these studies did not confirm specificity of their assay by comparison with an accepted method.

Purification of Urine Samples by Anion-Exchange Chromatography Prior to Assay of Iodide
For the ISE method to become widely used in the clinical setting, it must be reliable at all levels of urinary iodide, down to levels observed in severe I deficiency (<0.025 mg/L). To our knowledge, a procedure combining prior chromatographic purification to improve specificity and sensitivity of the ISE assay of iodide in biological fluids has not been published. In our opinion, purification prior to ISE measurement is a *sine qua non* requirement for specificity and sensitivity of the ISE assay under all physiological and pathological conditions including severe I deficiency (urine I <0.025 mg/L). Chromatographic purification of urine samples on columns of anion-exchange resin was selected for the reasons described below.

In 1962, Murthy, *et al,*[25] reported a procedure for removing radioactive iodide from milk. A strong anion exchange resin was used (Dowex 2 x 8) to retain the radioactive iodide, and a large volume of milk could be processed through these columns. A mean ± SD of 98±2% of the iodide could be retained on the columns when 120 bed volumes were chromatographed. The bed volume is approximately 1 ml per gm of resin. They were able to elute approximately 98% of the radioactive iodide from the column with 30 bed volumes of 2N sodium nitrate ($NaNO_3$) in 0.16N HNO_3. This publication was of great interest to us since the ISE assay of urinary iodide published by the Japanese scientists[20,21] used 5 ml of 5N $NaNO_3$ as ISA, added to 10 ml of urine. We postulated that by prior chromatographic purification of urine samples on anion exchange resin and using 5N $NaNO_3$ as the elution solvent to elute iodide, measurement of iodide could be made directly after adding two volumes of water to the eluant.
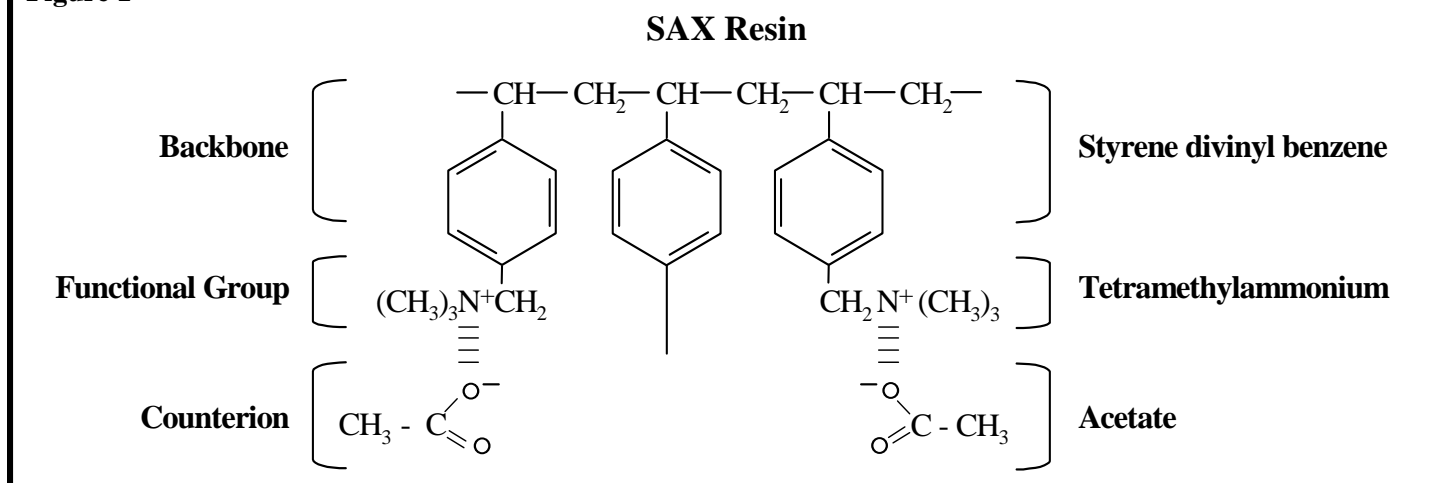
Materials used in anion exchange chromatography are composed of three components attached together and placed in a column: the base or backbone support; the functional group or ion exchanger; and the counter ion available for exchange. For backbone, styrene divinyl benzene (SDB) was preferred over silica gel because it is more rugged, less sensitive to pH changes, and possesses a higher capacity.[26] For example, SAX columns with silica backbone are available from Varian and Associates (Harbor City, CA) with a capacity of 0.85 m Eq/gm of resin. However, with SDB backbone, Alltech (Deerfield, IL) quotes a figure of 1.5 m Eq/gm, a 75% greater capacity to exchange anions. This translates into the ability to process a 75% greater volume of urine for the same amount of resin.

Strong anion exchangers are quaternary amines versus weak anion exchangers, which are primary, secondary, and tertiary amines. Strong anion exchangers are always charged at any pH; therefore, elution of the analate of interest could be achieved by increasing the ionic strength of the elution solvent without any acid added. Data are available for strong anion exchangers regarding the relative selectivity of halides.[26] With fluoride as unity, chloride has a relative selectivity of 10; bromide 28; and iodide 87. The higher the number, the stronger the binding of the halide to the anion exchanger. The stronger the binding of the halide to the ion exchanger, the higher the ionic strength required for elution. Therefore, by choosing a wash solvent with ionic strength high enough to elute fluoride, chloride, and bromine, but not high enough to elute iodide, a high degree of purity of the iodide fraction could be achieved.

For counterions, the choice from products available commercially was between chloride and acetate. Since chloride interferes in the assay, we chose the acetate form (Figure 1). Although a wide range of counterions could be prepared by preconditioning the anion exchange columns, this would not be practical in a clinical setting. The ideal counterion on the SAX columns used for the purification of iodide from the other halides would be nitrate. Based on information supplied by Alltech with the SAX columns, iodide is the only halide capable of displacing nitrate from the tetramethyl ammonium group. The smaller pore size of 60Å was preferable because it excluded molecules with molecular

Figure 1

SAX Resin

Backbone — Styrene divinyl benzene

Functional Group — Tetramethylammonium

Counterion — Acetate

Chemical structure of the SAX resin used in the chromatographic purification of urine samples prior to ISE measurement.

weight above 1,000, and it prevented overloading the resin with high molecular weight anions present in urine samples. Finally, the larger particle size was chosen because it allowed elution at the proper flow rate with lower pressure and vacuum. In Table 3 are displayed the various options commercially available for anion exchange chromatography. The characteristics chosen are in the right column.

The strong anion exchanger SAX was obtained from Alltech and tested with bed weights of 100, 200, 500, and 600 mg of SAX. The bed weights of 300 and 400 mg were not available commercially. This resin has a SDB base with a functional group of tetramethyl ammonium and with acetate as counterion (Figure 1). We were interested in a column of SAX that had the capacity to handle up to 30 ml of urine without overload and breakthrough of iodide. The reason for this will be explained later. Preliminary tests revealed that 500 mg of SAX was the minimum bed weight required to process 10-30 ml of urine without overload (breakthrough) of the column. Overloading the column resulted in the presence of iodide in the urine eluate after the first pass, causing low recovery of iodide in the purified fraction eluted with the high ionic strength solvent.

The 500 mg column with a 10 ml reservoir was chosen (Alltech part #309750). Ten milliliters of urine was applied to the SAX column, which was fixed on top of a vacuum manifold (Applied Separation Inc., Allentown, PA), connected to a vacuum pump (Alltech). We used the Bench Top Vacuum Station, which was capable of maintaining a preset vacuum. A vacuum of only two inches (50 mm) of mercury was sufficient for an elution flow rate of 4-5 ml/min. Due to variation in airflow through the different openings of the vacuum manifold

and variation of elution flow rates between the columns, there was a twofold difference between columns with the fastest and slowest flow rate. The elution of 10 ml of urine required 2-4 minutes at the vacuum setting of 50 mm Hg.

The four halides — fluoride, chloride, bromide, and iodide — were added individually in known amounts from stock standard solutions to pooled urine samples collected from a fasting subject. Using Thermo-Orion ISE
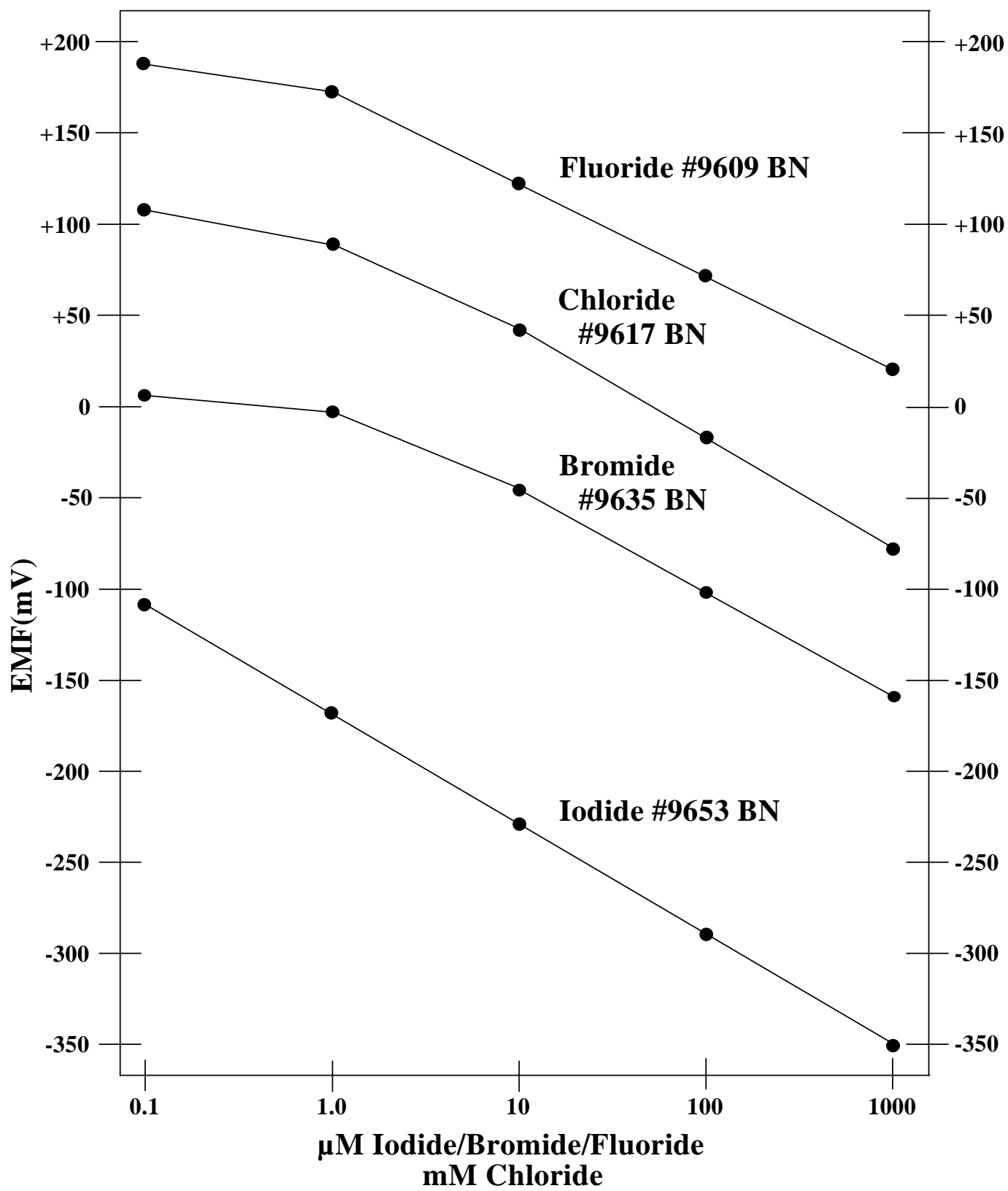
(Continued on next page)

Table 3

Options Commercially Available For Anion-Exchange Chromatography of Urine Samples*

I. Base (Backbone)	Silica Gel	Styrene-divinyl Benzene
II. Functional Group	Weak Exchanger (Primary, Secondary, and Tertiary Amine)	Strong Exchanger (Quarternary Amine)
III Counter-Ion	Chloride	Acetate
IV. Pore Size (Average Value)	Large (300 Å)	Small (60 Å)
V. Particle Size (Average Value)	Small (12 Microns)	Large (60 Microns)

*Characteristics chosen are in the right column

Figure 2

Dose-Response Curve for the Four Halides Using Thermo Orion ISE Electrodes

Fluoride #9609 BN

Chloride #9617 BN

Bromide #9635 BN

Iodide #9653 BN

EMF(mV)

μM Iodide/Bromide/Fluoride
mM Chloride

The Thermo Orion part number is displayed next to the standard curve.

electrodes and special reagents, the halides were measured following chromatography in the eluted urine, in the wash solvent (10 ml of 0.5N NaNO$_3$), and in the elution solvent (5 ml of 5N NaNO$_3$). The standard curves for the four halides are displayed in Figure 2.

The eluted urine contained fluoride and 75-80% of the chloride. Some 20-25% of the chloride was retained on the column, together with bromide and iodide. A wash of the column with 10 ml of 0.5N NaNO$_3$ eluted the retained chloride and the bromide. Quantitative recovery of iodide (>95%) was achieved with 5 ml 5N NaNO$_3$. The eluant containing the iodide in 5 ml of 5N NaNO$_3$ was mixed with 10 ml of water and measured directly by immersion of the iodide selective electrode (Orion Electrode #9653 BN) connected to the Orion Meter 720-A Plus. Standards of potassium iodide (Spectrum Chemical, Gardena, CA #P0185) were prepared in water at concentrations ranging from 10^{-3} M to 10^{-8} M. A volume of 10 ml of iodide standard was mixed with 5 ml of 5N NaNO$_3$ prior to measurement by the iodide selective electrode. The stored standard curve in the 720-A Plus meter was programmed to display the urinary I levels in mg/L.

Methodology
Urine samples collected over a period of 24 hours were stored by the subject during collection in a three-liter plastic bottle, supplied by Doctor's Data, Inc. After measurement of the total volume at the clinic, sodium azide was added at a final concentration of 0.05% for bacteriostatic purpose (10 ml of a 5% solution per liter of urine). Sodium azide is the commonly used bacteriostatic agent in such cases.[20] Prior to addition of the sodium azide, a sample was obtained and mailed to Doctor's Data for analysis by ICP-MS. Samples of the collected urine were then stored at -20° C in plastic containers until assayed by the ISE method. Repeated freezing and thawing of the urine samples had no significant effect on the measured I levels. However, without a bacteriostatic agent, such manipulation of the samples resulted in decreasing I levels and evidence of bacterial growth. Aqueous solutions of 0.5N and 5N NaNO$_3$ (Spectrum Chemical #SO183) were prepared and stored at room temperature. A stock solution of potassium iodide 1.66 gm in 1 liter of purified water (10^{-2} Molar) was stored in a dark glass bottle. From this stock solution, iodide standards were prepared by dilution to contain a range from 10^{-3} M (127 mg/L) to 10^{-8} M (0.00127 mg/L).

Under a vacuum of 40-/50 mm Hg, 10 ml of urine was applied to a SAX column 500 mg, with a 10 ml reservoir (Alltech #309750) and the elution flow rate adjusted so

Figure 3

FD & C Green No. 3 (Brilliant Green)
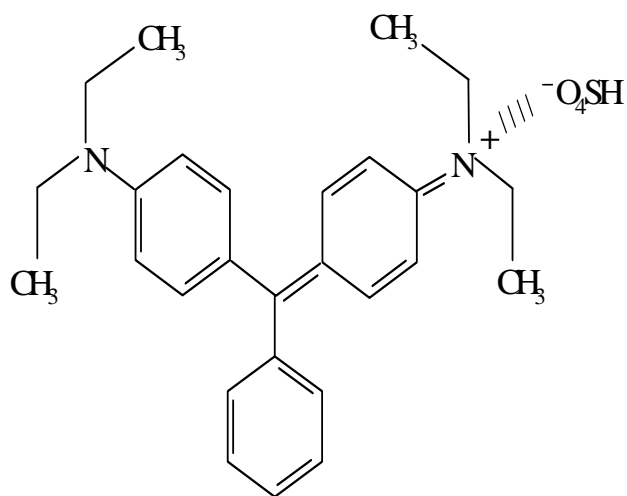
Chemical structure of Brilliant Green. (Modified from reference 27.) Note the two benzene rings and the quaternary amine group.

not to exceed 4-5 ml/min. This is the most critical step in the assay. A slower flow rate did not have an adverse effect on the performance of the anion-exchange columns. Exceeding this flow rate, however, caused breakthrough of iodide in the urine eluate, with low recovery of iodide in the assay. At the same vacuum setting, the elution flow rate decreased with the wash and elution solvents. Therefore, it was very important to set the flow rate during the elution of the urine sample. Since there was variation in flow rate between the columns, the SAX column with the highest flow rate was used to monitor visually the urine level in the reservoir. To facilitate this process of observing urine levels through the opalescent wall of the reservoir, a food coloring was added to the urine samples prior to chromatography. FD & C Green No. 3 (Warner Jenkinson Company, St. Louis, MO) was selected for its useful attributes. Having two benzene rings in its molecule (Figure 3) resulted in a very strong hydrophobic interaction with the benzene rings in the SDB backbone of the SAX (Figure 1). FD & C Green No. 3 was retained on the column, resulting in a clear eluate. Although FD & C Green No. 3 contains a quaternary amine (Figure 3) capable of binding iodide, its concentration was so low that no significant interference was observed in the performance of the columns. We added 1 ml of a 1% solution per liter of urine. The amount of that dye in 10 ml of urine was 0.2 µMole, compared to a concentration of 750 µMoles of

(Continued on next page)

quaternary amines on the SAX column. FD & C Green No. 3 has antiseptic, therefore bacteriostatic properties, since it is used topically and orally in veterinary medicine as an antiseptic.[27] We are currently testing FD & C Green No. 3 as a bacteriostatic agent to replace sodium azide. So far, the results look very promising.

We have tested several SAX columns with both silica and SDB backbones obtained from Alltech and Varian. The vacuum setting required for the proper elution flow rate varied widely between SAX columns with as much as 15 inches (375 mm) Hg for some SAX columns with silica backbone and small particle sizes. With the Alltech 500 mg SAX columns, however, a vacuum setting of 50 mm Hg resulted consistently in the proper flow rate. The Alltech vacuum pump displayed the ambient atmospheric pressure in mm Hg. The desired vacuum was achieved by setting the vacuum pump at 50 mm below ambient.

Chromatography of the urine sample on the SAX column yielded three fractions, which were used to measure fluoride, bromide, and iodide. The flow chart in Figure 4 summarizes this procedure. The eluted urine contains >95% of the fluoride and 75-80% of chloride. Ten milliliters of the special ISA TISAB II (Thermo Orion #940909) was added to the 10 ml of eluted urine, and fluoride level was measured with electrode #9609BN. The wash solvent consisted of 10 ml of 0.5N $NaNO_3$ and following chromatography on the SAX column contained 20-25% chloride and >95% bromide. Using the electrode #9635BN, bromide levels were measured following addition of 5 ml of 5N $NaNO_3$ to the eluted wash solvent. The bromide standards used to compute the standard curve were prepared in 0.5N $NaNO_3$. Validation of the bromide assay will be the subject of another report. The last step was the addition of 5 ml of 5N $NaNO_3$ to the column and elution of iodide at the same vacuum setting, although the flow rate was less, between 2-4 ml/min. Ten milliliters of water was mixed with the eluted 5 ml 5N $NaNO_3$. Iodide concentration was measured directly by immersion of the electrode (Orion #9653 BN).

Acceptability of the ISE Method for Urinary Iodide Measurement
We followed the same procedure we previously described for the validation of radioimmunoassay of steroid hormones in biological fluids.[28,29] The criteria for acceptability of an assay includes reliability and practicability. The reliability of an assay depends on its sensitivity, specificity, accuracy, and precision. The practicability of an assay is judged by the skill required to per-

(Continued on next page)

Figure 4

Flowchart Describing the Procedure Used in the Combined Measurement of Fluoride, Bromide, and Iodide in the Same Urine Sample
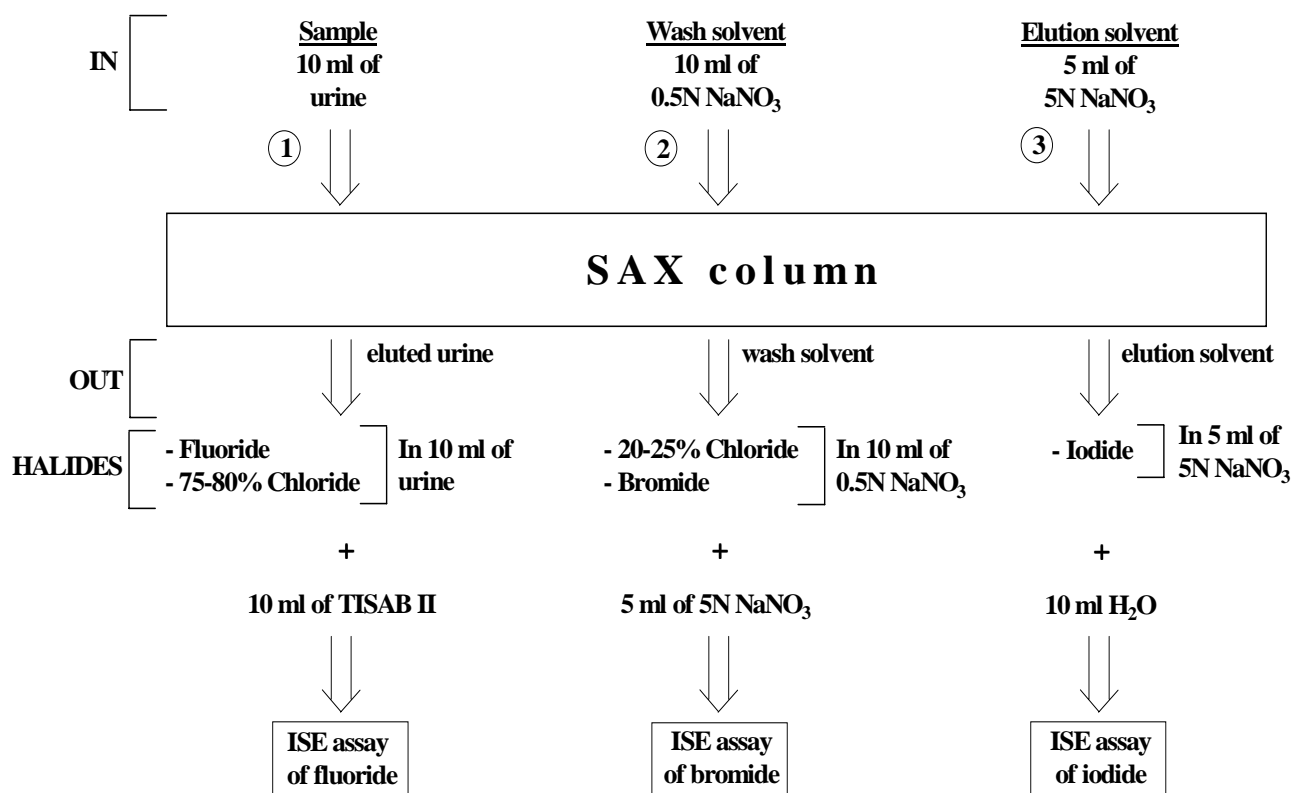
form it, the time involved in its performance, and the cost of the assay.

Reliability eExperiments

*Sensitivity***:** The theoretical limit of sensitivity achievable with ISE assay of urinary iodide is set by the sensitivity of the iodide selective electrode itself. From data supplied by Thermo Orion, the sensitivities of the ISE electrodes for halides are: for iodide, 5×10^{-8} M; for fluoride, 10^{-6} M; for bromide, 5×10^{-6} M; and for chloride, 5×10^{-5} M. The iodide selective electrode is by far the most sensitive, being 20 times more sensitive than the ISE electrode for fluoride, 100 time more sensitive than for bromide, and 1,000 times more sensitive than for chloride. Expressed as mg/L, the iodide selective electrode is sensitive down to 0.006 mg/L, compared to a sensitivity of 0.003 mg/L, for ICP-MS used by Doctor's Data Laboratories. (Information on sensitivity of ICP-MS supplied by Dean Bass).

In achieving this theoretical sensitivity, other conditions are important. First, the sensitivity of the standard curve is a limiting factor. The sensitivity of the standard curve is defined as the smallest amount of iodide that is significantly different from zero at the 95% confidence limit. In order to compute the standard curve (the dose response curve), the EMF expressed in millivolts (mV) was plotted on the Y-axis against the logarithm of increasing amount of iodide, from 10^{-3} M to 10^{-8} M on the X-axis. The iodide selective electrode was extremely sensitive (Figure 5), with a linear response from 10^{-3} M to 10^{-7} M. A mean D EMF of 61 mV per decade was observed from 10^{-3} M to 10^{-7} M, but from 10^{-7} M to 10^{-8} M, the standard curve became non-linear with only 36.2 mV per decade.

To calculate the mean blank value, samples containing zero iodide are run in the assay using several replicates, ideally six. The sensitivity would then be equal to two standard deviations from the mean blank value, after subtracting the mean blank value.   We tested water blanks and deiodized urine blanks. When six samples of water of 10 ml each were chromatographed on the SAX column as described under methodology, we obtained a mean $\pm$ SD of 0.0024 $\pm$ 0.0006 mg/L. Deiodized urine

Figure 5

Dose-Response Curve Observed with the Iodide-Selective Electrode

Meter: ORION #720 - A Plus
Electrode = ORION #9653 BN
Filling Solution = ORION #D
ISA = NaNO$_3$ 5M
Standard Volume/ISA Volume = 2

µM	EMF (mV)	@ EMF (mV)
0.01	- 72.4	
0.1	-108.6	36.2
1.0	-170.7	62.1
10	-232.7	62.0
100	-292.9	60.2
1000	-352.5	59.6

EMF (mV)

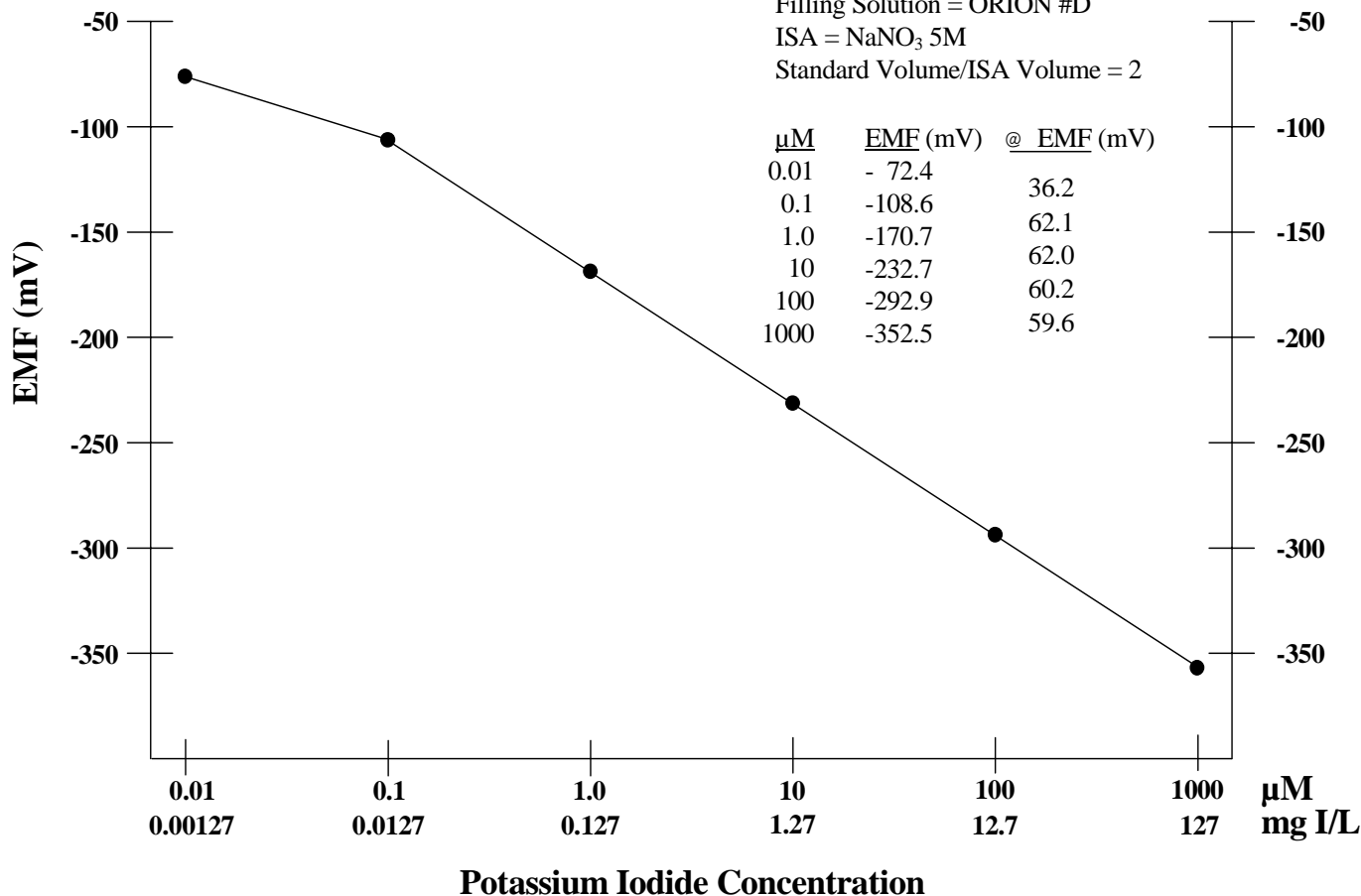|	0.01	0.1	1.0	10	100	1000	**µM**
	0.00127	0.0127	0.127	1.27	12.7	127	**mg I/L**

Potassium Iodide Concentration

Table 4

Correlation Between Urinary I Levels Measured by ICP-MS with Levels Obtained by the ISE Method

Sample Number	Iodoral® # Tab	mg I/24 hr	
		ICP-MS	ISE
1	1	2.7	3.2
2	1	2.6	3.3
3	1	3.1	2.8
4	1	2.6	2.5
5	1	3.3	4.5
6	1	2.5	2.8
7	2	6.0	7.2
8	2	6.5	6.8
9	2	5.4	6.2
10	3	14.0	19.0
11	3	9.9	10.7
12	3	6.8	9.0
13	3	11 (16)*	18.2
14	3	7.7	9.2
15	3	7.5	10.6

*Repeat measurement by ICP-MS

was prepared as described by Cooper and Croxson.[19] The 95% confidence limits of the mean blank were: 0.003-0.005 mg/L. Based on this information, a sensitivity of 0.005 mg/L could be achieved, a value very close to the 0.006 mg/L suggested by Thermo Orion. This sensitivity, however, could be improved by three-fold, using 30 ml of urine for chromatography, but keeping the wash solvent volume at 10 ml and elution solvent volume at 5 ml. Using 30 ml of urine, the sensitivity in measuring I in urine samples by the ISE method was 0.0017 mg/L, comparable to the sensitivity of ICP-MS.

Specificity: There are various ways of validating an assay in terms of its specificity, one of which is by comparison with an accepted method. Yabu, *et al*,[19] and Kono, *et al*,[20] validated the specificity of the ISE method for direct measurement of urinary iodide levels by comparison with the ceric ion-arsenious acid method.[21]

However, their direct assay without prior purification proved unreliable with urinary I levels below 1.27 mg/L. In our US population, based on the latest nutritional survey, only 5% of urine samples evaluated had I levels above 0.5 mg/L.[1] Therefore, the direct ISE method for urinary I levels, although acceptable for mainland Japanese, would be worthless in our population. By prior purification of the urine samples using anion-exchange chromatography, we were able to achieve a sensitivity 250 times better than that reported by the Japanese authors, and 750 times better if 30 ml of urine were chromatographed on the SAX column.
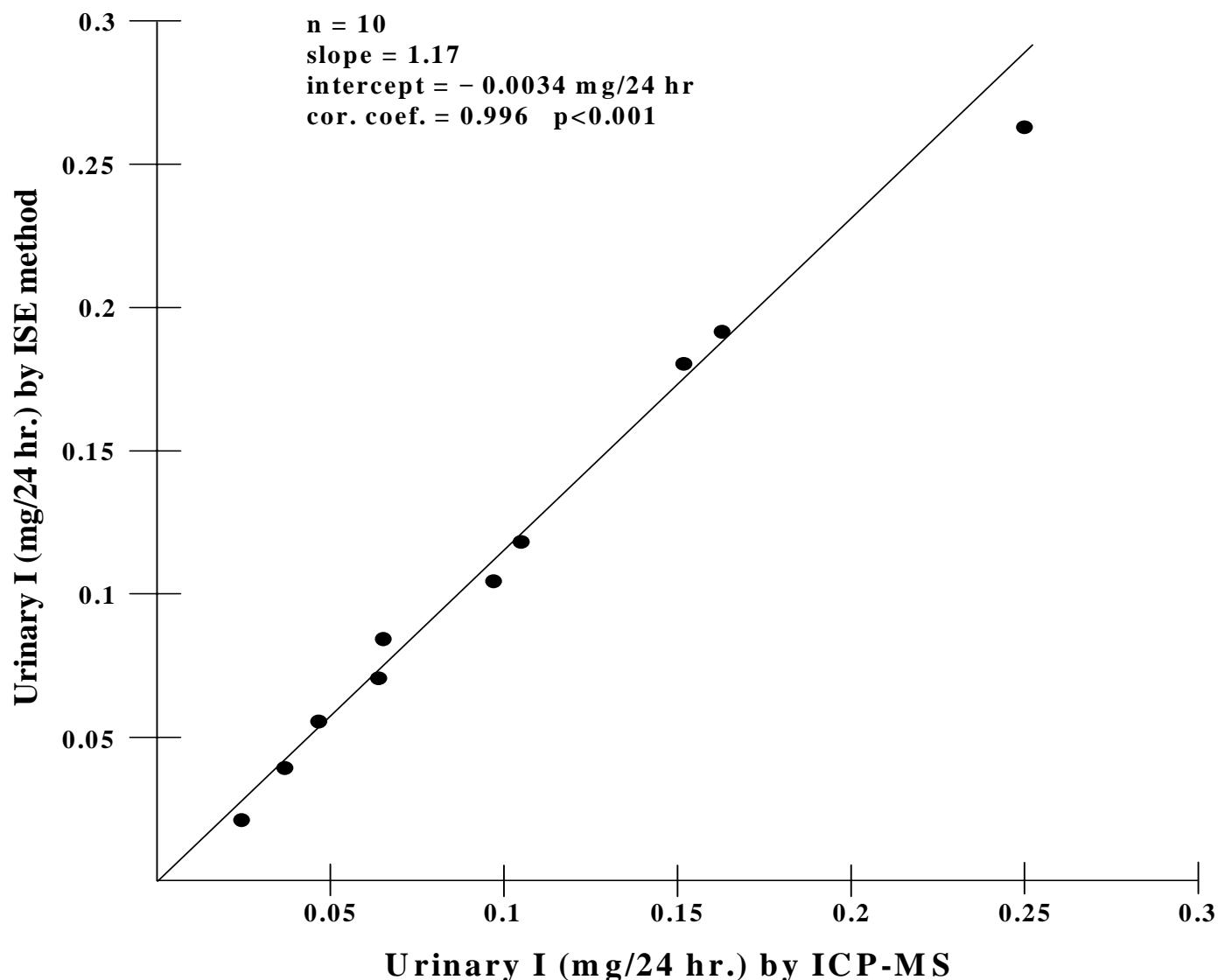
To validate the specificity of our procedure, we chose the ICP-MS as the valid, accepted method. From the data presented in Tables 1 and 2, urine samples were available for comparison from 10 subjects (one sample per subject) for baseline I levels, and urine samples from the six subjects in Table 2 were used for the post-I supplementation comparison of the ISE method with ICP-MS. Out of 18 samples analyzed by ICP-MS in those six subjects following I-supplementation, 15 samples were available for comparison with the ICP-MS method. With baseline urinary I levels between 0.022-0.25 mg/24 hr in the 10 samples used for comparison (Figure 6), a correlation coefficient of 0.996 ($p<0.001$) was obtained. For urinary I levels expected in the US population, the ISE method described in this manuscript is therefore a reliable assay and the cost of setting up the ISE procedure is within the reach of the average clinician.

We have not achieved as good a correlation with ICP-MS, using urine samples with I levels in the range observed in mainland Japanese, that is 100-fold higher values. Urinary I levels following three tablets of Iodoral® were consistently higher by the ISE method, being as much as 65% higher than reported by ICP-MS. For example, in sample #13 (Table 4), a value of 11 mg I/24 hr was reported by ICP-MS following three tablets of Iodoral®. We measured 18.2 mg I/24 hr in the same sample. Another sample from the same urine collection was sent to Doctor's Data, but labeled as a new sample. The repeat value was 16 mg I/24 hr. One of us (GEA) talked to Dean Bass, who was very helpful. He explained that his equipment is calibrated to measure I levels within the range expected in the U.S. population, that is from 0.012 to 0.5 mg/24 hr. The accuracy of their assay, however, will not be as good with I values 100-fold higher, unless the urine samples were diluted 100-fold. He exhorted us to share this information with other physicians who plan to use Doctor's Data for I analysis and who are using our program. Clearly mark on urine samples "High iodide levels!! Dilute 100-fold prior to measurement." We

(Continued on next page)

Figure 6

**Correlation Between Urinary I Levels Obtained by ICP-MS
with Values Obtained by the ISE Method**

n = 10
slope = 1.17
intercept = − 0.0034 mg/24 hr
cor. coef. = 0.996   p<0.001

Urinary I (mg/24 hr.) by ICP-MS

have not observed this carryover effect even with a difference of 1,000-fold between urine I levels of consecutive samples, using the ISE method and carefully washing the electrode between measurements.

Accuracy: The accuracy of the ISE method was tested by recovery experiments. To deiodized urine was added an increasing amount of potassium iodide from 0.01 µM to 100 µM, and measurement of I was performed in five replicates at each dose level. The recovery experiment was performed in the same batch of samples. The mean percent recovery over the range tested varied from 91% to 112% (Table 5).

Precision: The within-assay variance was tested by per-

forming five replicate analyses of three urine samples, one sample with baseline I level and two samples following I supplementation. For the between-assay variance, these three urine samples were measured on five consecutive days.   The coefficient of variation was higher for between assay than within assay, with a range of 5.7-10.5% for within assay and a range of 10.5-18.0% for between assay precision (Table 6).

Practicability
As previously mentioned, the practicability of an assay depends on the degree of skill required to perform it, the time involved in its performance, and the cost of the assay.

Table 5

Recovery of Added Potassium Iodide to Deiodized Urine
Using the ISE Method of Measurement

Potassium Iodide Added		Iodide Measured	
μM	mg I/L	mg I/L	Mean % Recovery
0	0	<0.005	—
0.01	0.00127	<0.005	—
0.1	0.0127	0.0145 ± 0.0035	110%
1	0.127	0.116 ± 0.028	91%
10	1.27	1.38 ± 0.26	109%
100	12.7	14.2 ± 1.65	112%

Skill: First, we should mention that the Clinical Laboratory Improvement Amendments of 1988 (CLIA) defined two categories of complexity for laboratory test: moderate complexity and high complexity testing. The ISE method is only 20 years old and is used currently in university and research laboratories, and rarely in clinical laboratories. For example, Doctor's Data, Inc. performs urinary fluoride levels by the ISE method, and it is classified as high complexity testing mainly because the ISE procedure is not widely used in the clinical context, at least not in the US. It is likely that with increased applications to clinical testing, the ISE method will be downgraded to moderate complexity.

Obviously, familiarity with laboratory equipment such as vacuum manifold, solid phase extraction, and potentionmetric measuring devices is a basic requirement for the analyst involved in ISE measurement. Although no special skill is required for implementation of this procedure in a physician's office, meticulous attention to cleanliness, awareness of the possible sources of interference, and consistency in the performance of the ISE method are qualities that the technician involved in ISE measurement must have. A clearly written protocol and well-defined guidelines of quality control should not be difficult to comply with, mainly in a clinic laboratory already approved for moderate complexity procedures.

Time Involved: Vacuum manifolds are available from Applied Separations, Inc., with 30 positions, so that chromatography of 30 samples could be performed in one batch. The ISE measurement of iodide is relatively rapid, usually less than five minutes per sample. This procedure lends itself to automation, and equipment is commercially available for this purpose. We have just

Table 6

Within and Between Assay Variances in the Measurement
of Urinary I Levels by the ISE Method

Sample	ISE Method (mg/L)			
	Within Assay (5)		Between Assay (5)	
	$\overline{X} \pm SD$	CV*	$\overline{X} \pm SD$	CV
1	0.035 ± 0.002	5.7%	0.038 ± 0.055	14.5%
2	1.1 ± 0.08	8.0%	1.32 ± 0.24	18.0%
3	4.1 ± 0.43	10.5%	4.4 ± 0.55	10.5%

* CV = Coefficient of variation.
( ) = Number of replicate measurements.

acquired a new model from Thermo Orion, the #960 Meter with a 45-position Autosampler. This minimizes human intervention and, therefore, human error. It is of interest to note that using the 960 Model, the sensitivity is calculated by proprietary software in the unit, without human intervention. Surprisingly, the computed sensitivity for I determination, using the same electrode, was 0.006 mg/L, the same value we obtained, as described previously with the model 720 A-Plus meter. The upgrade from semiautomation to full automation could be justified with increased number of samples, such as in a Polyclinic or commercial laboratory.

Cost: The 720 A Plus meter, with iodide-selective electrode and printer, the 30-position vacuum manifold, the computerized vacuum pump, and the usual laboratory glassware and pipetting devices would require an initial investment of $5,000. We are currently preparing a list of equipment and other items necessary to get started in a physician's office laboratory already approved by CLIA for moderate complexity testing. Check with your local CLIA representative and your clinical pathologist for the requirements.

Discussion
Methodology: Since the discovery of iodine/iodide in the early 1800s, the measurement of this element has progressed steadily from colorimetry to the present mass-spectrometry. In the US, the starch-iodide reaction was the detection method in the 1930s,[31] replaced with the ceric ion-arsenious acid of the 1940s.[32] This last procedure is still used today in most clinical laboratories, where it has been automated.[22] The two latest additions over the last 20 years have been the ISE method[20,21,23,24] and ICP-MS, recently introduced in some clinical laboratories. The advantage of ICP-MS is the ability to measure a large number of elements in the same biological sample. Its disadvantage is the astronomical cost of acquisition and maintenance.

The ISE procedure for measuring iodide is very simple, using non-toxic reagents, and yielding results within minutes. In Japan, the ISE method can be used directly in urine samples.[20,21] However, in the US, the level of sensitivity of the ISE assay, if used directly, is 100 times lower than urine I levels usually present in the US population. Recently, the availability of SAX resin, with the purity required for purification of very low levels of iodide, made it possible to apply the ISE method to the measurement of urinary I levels in the US population. To our knowledge, this is the first time the purification of biological fluids on SAX columns, followed by measurement of iodide by the ISE method, is reported. Ideally, all equipment, supplies and reagents needed for the

ISE assay of urinary I levels should be available from one source, and this will most likely happen with increased demand. There is a need for a central laboratory capable of supplying urine samples with known amounts of iodide in order to monitor the precision and accuracy of the assay of urinary iodide in the physician's office and in commercial laboratories. We are concerned about the great discrepancy observed with values of urinary I levels reported by commercial laboratories using the same procedure, ICP-MS. Samples from the same urine collection were mailed to Doctor's Data and to Mayo Medical Lab (Wilmington, MA). The value reported by Doctor's Data was 11 mg I/24 hr, but Mayo Medical reported 99 mg I/24hr for the same sample, a nine-fold difference.

Clinical Applications: It is estimated that a third of mankind suffers from I deficiency, defined by the World Health Organization as urinary I levels below 0.05 mg/L.[1] However, I sufficiency to prevent simple goiter and cretinism was considered adequate. I sufficiency of the whole human body has never been studied. Based on a review of published studies, we previously proposed that an amount of I about 100 times the RDA would be required for I sufficiency of the whole human body.[13,30] Using this new definition of I sufficiency, only mainland Japanese consume adequate levels of I, with 99% of the world suffering from I deficiency.

There is a great need for a simple test to assess I sufficiency of the whole human body. The I-loading test mentioned in this manuscript, using three tablets of Iodoral®, may be adequate in a clinical setting. Correlation of percentage I retained with clinical improvement of such conditions as FDB could be used to fine tune this I-loading test. Ghent, *et al*,[5] have suggested that the amount of I needed in women was dependent on their body weight. The preliminary data presented in this manuscript suggest that breast size and pathology may also play an important role in I requirement by the whole human body.

The high prevalence of I deficiency in the adult female US population[1] justifies the routine measurement of urinary I levels in every female patient evaluated in clinical practice. The procedure described in this manuscript makes it possible to perform such measurements in the physician's office.

About the Author

Guy E. Abraham, MD, is a former Professor of Obstetrics, Gynecology and Endocrinology at the UCLA

School of Medicine. Some 35 years ago, he pioneered the development of assays to measure minute quantities of steroid hormones in biological fluids. He has been honored as follows: General Diagnostic Award from the Canadian Association of Clinical Chemists, 1974; the "Medaille d'Honneur" from the University of Liege, Belgium, 1976; the Senior Investigator Award of Pharmacia, Sweden, 1980.

The applications of Dr. Abraham's techniques to a variety of female disorders have brought a notable improvement to the understanding and management of these disorders. Twenty-five years ago, Dr. Abraham developed nutritional programs for women with premenstrual tension syndrome and post-menopausal osteoporosis. They are now the most commonly used dietary programs by American obstetricians and gynecologists. Dr. Abraham's current research interests include the development of assays for the measurement of iodide and the other halides in biological fluids and their applications to the implementation of orthoiodosupplementation in medical practice.

J. C. Hakala, RPh, is a registered pharmacist who specializes in compounding nutritional and pharmaceutical products for physicians. He is the founder/owner of Hakala Apothecaries.

Jorge D. Flechas, MD MPH, is the medical director of Flechas Family Practice in Hendersonville, North Carolina, specializing in hormonal therapy for treatment of fibromyalgia and chronic fatigue and immune dysfunction syndrome (CFIDS) since the late 1980s. He also specializes in iodine therapy for hypothyroidism and fibrocystic breast disease.

REFERENCES

1) Hollowell J, Staehling N, *et al*. "Iodine nutrition in the United States. Trends and public health implications: Iodine excretion data from national health and nutrition examination surveys I and III (1971-1974 and 1988-1994)." *J Clinical Endocrinology and Metabolism*, 1998; 83:3401-3408.
2) Eskin B, Bartuska D, *et al*. "Mammary gland dysplasia in iodine deficiency." *JAMA*, 1967; 200:115-119.
3) Eskin B. "Iodine metabolism and breast cancer." *Trans New York Acad of Sciences*, 1970; 32:911-947.
4) Funahashi H, Imaj T, *et al*. "Suppressive effect of iodine on DMBA-induced breast tumor growth in the rat." *Journal of Surgical Oncology*, 1996; 61:209-213.
5) Ghent W, Eskin B, *et al*. "Iodine replacement in fibrocystic disease of the breast." *Can J Surg*, 1993; 36:453-460.
6) Derry D. *Breast Cancer and Iodine*. Trafford Publishing, Victoria, BC, 2001; 92.
7) Vishnyakova VV and Murav'yeva NL. "On the treatment of dyshormonal hyperplasia of mammary glands." *Vestn Akad Med Navk SSSR*, 1966; 21:19-22.
8) Cann S, Netten J, and Netten C. "Hypothesis: Iodine, selenium and the development of breast cancer." *Cancer Causes and Control*, 2000; 11:121-127.
9) Ghandrakant C, Kapdim MD, and Wolfe JN. "Breast cancer. Relationship to thyroid supplements for hypothyroidism." *JAMA*, 1976; 238:1124.
10) Epstein SS and Steinman D. *Breast Cancer Prevention Program*. Macmillan, New York, 1998; 5.
11) Waterhouse J, Shanmvgakatnam K, *et al*. *Cancer Incidence in Five Continents*. LARC Scientific Publications, International Agency for Research on Cancer, Lyon, France, 1982.
12) Stadel B. "Dietary iodine and risk of breast, endometrial, and ovarian cancer." *The Lancet*, 1976; 1:890-891.
13) Abraham GE, Flechas JD, and Hakala JC. "Optimum levels of iodine for greatest mental and physical health." *The Original Internist*, 2002; 9(3):5-20.
14) Gennaro AR. *Remington: The Science and Practice of Pharmacy, 19th edition*. Mack Publishing Co, 1995; 1267.
15) Plummer HS. "Results of administering iodine to patients having exophthalmic goiter." *JAMA*, 1923; 80:1955.
16) Thompson WO, Brailey AG, *et al*. "The range of effective iodine dosage in exophthalmic goiter." *Arch Int Med*, 1930; 45:261-281.
17) Follis RH, Vanprage K, and Damrougnakdi D. "Studies on iodine nutrition in Thailand." *J Nutr*, 1962; 76:159.
18) Follis RH. "Patterns of urinary iodine excretion in goitrous and nongoitrous areas." *Amer J Clin Nutr*, 1964; 14:258.
19) Cooper G and Croxson MS. "Chloride interference with use of an iodide-selective electrode for urinary I." *Clinical Chemistry*, 1983; 29:1320.
20) Yabu Y, Miyai K, *et al*. "Measurement of iodide in urine using the iodide selective ion electrode." *Endocrinology*, 1986; 83:905-911.
21) Konno N, Yuri K, and Miura K. "Clinical evaluation of the iodide/creatinine ratio of casual urine samples as an index of daily iodide excretion in a population study." *Endocrine Journal*, 1993; 40:163-169.
22) Garry PJ, Lashley DW, and Owen GM. "Automated measurement of urinary iodine." *Clin Chem*, 1973; 19:950-953.
23) Lacroix DE and Wong P. "Determination of iodide in milk using the iodide specific ion electrode and its application to market milk samples." *Journal of Food Protection*, 1980; 43:672-674.
24) Gushurst CA, Mueller JA, *et al*. "Breast milk iodide: Reassessment in the 1980s." *Pediatrics*, 1984; 73:354.
25) Murthy GK, Gilohrisk JE, and Campbell JE. "Method for removing iodine[131] from milk." *J Dairy Sci*, 1962; 45:1066-1074.
26) Thurman EM and Mills MS. *Solid-Phase Extraction. Principles and Practice*. Wiley-Interscience Publication, 1998; 123-160.
27) *The Merck Index, 13th edition*. Merck and Co, Inc, New Jersey, 2001; 230.
28) Abraham GE. "Radioimmunoassay of steroids in biological materials." In: *Radioimmunoassay and Related Procedures in Medicine*. Proceedings of a symposium held by the International Atomic Energy Agency, Vienna, Austria, 1974; 3-30.
29) Abraham GE. "The application of natural steroid radioimmunoassay to gynecologic endocrinology." In: *GE Abraham's Radioassay Systems in Clinical Endocrinology*, Marcel Dekker, Inc, 1981; 475-530.
30) Abraham GE, Flechas JD, and Hakala JC. "Orthoiodosupplementation: Iodine sufficiency of the whole human body." *The Original Internist*, 2002; 9(4):30-41.
31) Turner RG. "A micro colorimetric method for the quantitative estimation of iodine in blood." *J Biol Chem*, 1931; 88:497-511.
32) Taurog A and Chairoff IL. "On the determination of plasma iodine." *J Biol Chem*, 1946; 163:313-322. u