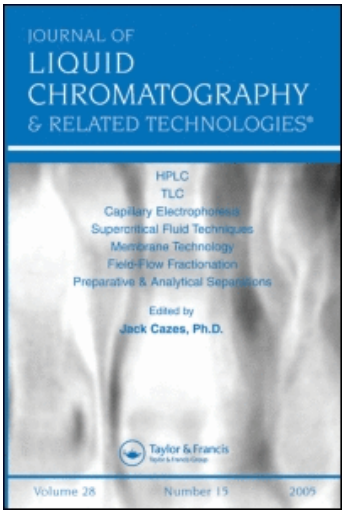

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SERUM INORGANIC IODIDE DETERMINED BY PAIRED-ION REVERSED-PHASE HPLC WITH ELECTROCHEMICAL DETECTION

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ABSTRACT

We propose an automated method for the analysis of serum inorganic iodide (SII), using paired-ion reversed phase HPLC with electrochemical detection and a silver working electrode. Assay conditions include a flow rate of 1.0 mL/min and an operating potential of 0.10 V. The retention time for iodide is 5.3 min. Sample preparation consists in protein removal by ultrafiltration and concentration of the ultrafiltrate, because of the very low levels of SII, especially in iodine deficient areas. Ultrafiltration separation is achieved by pouring 2mL of a serum sample into a filter cup (membrane cutoff: 5kD) and then using a centrifugal force of 14000G over 90 min. A 1200μL aliquot of the ultrafiltrate is concentrated by a factor of 10 to a volume of 120μL in a centrifugal vacuum concentrator (Speed-Vac). A 100μL aliquot of the concentrate is injected into the HPLC. Due to concentration the detection threshold (signal-to-noise ratio of 3) could be lowered to 0.004μmol/L. The recovery of iodide during concentration of the ultrafiltrate tested with ^{123}I was 102.2%.

The within and between-run precision (CV) for a serum sample with 0.04μmol/L are 1.9% and 4.2%, respectively. For comparison with a standard method based on the isotope dilution principle serum samples from 26 patients who underwent thyroid scintigraphy with ^{123}I were measured by both HPLC(y) and the standard technique(x). The data obtained show a high correlation (r=0.99; y=0.94x + 0.007; S_{yx}=0.0179). Levels of SII typical of an iodine deficient area are measured in sera from 27 patients with low urinary iodine excretion (44μmol/molCrea): SII=0.023μmol/L (mean); range: 0.01- 0.036μmol/L.

INTRODUCTION

Iodine deficiency is the most common cause of thyroid enlargement and goiter is, indeed, the obvious and familiar feature.[1] But it is only a visible mark of an inadequate iodine supply. Iodine deficiency disorders[2] is the term now used to denote all the effects of functional and developmental abnormalities, including thyroid disorders, which occur when the physiological requirements of iodine are not met in a given population. Evaluation of the status of iodine nutrition is therefore highly desirable.[3] As most of iodine is excreted in the urine, urinary iodine excretion is currently the most convenient laboratory marker of iodine deficiency.[4] The present recommendation[5] is to evaluate the iodine intake of a given population by the measurements of iodine concentrations in a representative number of casual urine samples.[6,7] The most precise estimation of the iodine supply of an individuum, however, is the determination of the urinary iodine in 24-hour collections.[8,9] The feasibility and the completeness of 24-hour collections however, are often in doubt, since accurate urine collections are notoriously difficult to obtain. The measurement of serum inorganic iodide (SII) therefore might be a more convenient alternative for demonstrating iodine deficiency of an individuum. Furthermore many aspects of iodine metabolism, especially the effects of iodine on the thyroid gland,[10] can only be fully understood if the concentration of SII is known. The determination of SII is, therefore, of great value in pathophysiologic studies of thyroid function, particularly for the calculation of the absolute iodine uptake.[11,12,13]

Here, we present a relatively simple automated method for the direct determination of inorganic iodide in serum using paired-ion, reversed phase HPLC and ultrafiltration for removal of proteins. The required sensitivity was achieved by concentrating the iodide content of the ultrafiltrates with a centrifugal vacuum concentrator (Speed-Vac).

MATERIALS AND METHODS

Apparatus

We assembled a modular HPLC system comprising a Model 510 HPLC pump, a Model 717 autosampler, a Model 460 electrochemical detector, and a temperature-control system consisting of the temperature-control module and a column heater (all from Waters Chromatography Div., Millipore, Milford, MA). Specific subcomponents of the electrochemical detector included a Ag/AgCl reference electrode, a 50μ gasket defining an analytical cell volume of 2.5μL and a silver working electrode.

The autosampler and detector were connected on-line to a Power Mate 386/25 personal computer(NEC Technologies, Boxborough, MA) operating with 810 Baseline chromatography software from Waters. The chromatographic column was a Resolve™ C_{18} reversed phase column, 3.9 x 150 mm, 90Å (9 nm), 5μm (Waters) held at a temperature of 35°C in the column heater.

Chemicals

Analytical-grade di-sodium hydrogen phosphate dodecahydrate ($Na_2HPO_4.12H_2O$), ethylene dinitrilo tetraacetic acid (Titriplex™ III) and H_3PO_4 were obtained from Merck(Darmstadt, Germany); di-n-butylamine was from Sigma Chemical CO. (St.Louis, MO). The ion-pairing reagent tetrabutylammonium phosphate(TBAP) was purchased from Waters and analytical-grade potassium iodide from Serva (Heidelberg, Germany) was used to prepare calibration solutions.

Mobile Phase

The mobile phase consisted of 10 mmol/L $Na_2HPO_4.12H_2O$, 1 mmol/L Titriplex III, 10 mmol/L TBAP, and 6 mmol/L di-n-butylamine in HPLC-grade water. The pH of the resulting mobile phase was adjusted to 7.0 with 85% orthophosphoric acid (H_3PO_4). Before use, the mobile phase was filtered through a 0.45-μm-pore membrane filter (Waters) and degassed under reduced pressure.

Serum Samples

Serum samples were collected from 27 euthyroid children (age 9-17y, mean 12.8y), living in an iodine deficient area with low urinary iodine excretion: 44 μmol/mol creatinine (median). For comparison with the standard method based on the isotope dilution principle (s.below), sera from 26 euthyroid patients referred to our clinic for scintigraphic evaluation of thyroid nodules were obtained.

Separation of proteins by ultrafiltration[14] was achieved by pouring 2mL of a serum sample into a filter cup (UFC4LCC25 from Nihon Millipore Kogyo,Japan) with a membrane cutoff of 5kD and, then, using a centrifugal force of 14000G over 90 min. A 1200μL aliquot of the ultrafiltrate is concentrated by a factor of 10 to a volume of 120μL in a Speed-Vac-concentrator. A 100μL aliquot is injected into the HPLC.

HPLC Analysis

We used isocratic HPLC at a flow rate of 1.0 mL/min with electrochemical detection at a potential difference of 0.10 Volt vs Ag/AgCl. The full-scale integrator sensitivity was 1.0 Volt, corresponding to a full-scale detector sensitivity of 50 nA. Concentrations were calculated from peak areas by using the integrator.

Comparison Method Based on the Isotope Dilution Principle

According to Stanley,[15] the calculation of serum inorganic iodide(SII) is based on the assumption that the specific activity of SII equals the specific activity of urinary inorganic iodide. Applying a simplified procedure with one blood sample only SII can, therefore, be determined from the following formula:[13,15,16]

SII = Serum ^{127}I= Serum^{123}I(1hr) x (Urinary^{127}I(1-2hr) / Urinary^{123}I(1-2hr)) where ^{127}I is stable iodine and ^{123}I is a radioactive isotope of iodine frequently used in thyroid scintigraphy.

Patients, 26, referred for scintigraphic evaluation of nodular goiter were studied. After each patient had emptied his bladder, a tracer dose of 10MBq (270μCi) of ^{123}I was administered intravenously. A blood sample was taken one hour after injection of ^{123}I for measurement of serum ^{123}I and urine collection was extended over two hours after ^{123}I administration for determination of urinary ^{123}I and urinary ^{127}I. The radioactivity of ^{123}I was

Table 1

Within and Between-Run Precision (Coefficients of Variation=CV) for Determination of Serum Inorganic Iodide Concentrations Using HPLC

Serum Sample (μmol/L)	Intraassay-CV (%)	Interassay-CV (%)
0.12	1.13	5.35
0.04	1.88	4.19
0.02	1.91	8.36

measured in a well-type counter, urinary ^{127}I was determined by HPLC.[17] SII was calculated according to the above mentioned formula and compared with the value obtained by measurement of SII in the same blood sample, using HPLC.

Statistical Analysis

Correlations between the results by HPLC and the reference method based on the isotope dilution principle were assessed with ordinary least-squares linear regression techniques. Intra- and interassay SDs and CVs (Table 1) were calculated from 10 measurements of three serum samples with different iodide concentrations. Differences between slopes measured by adding increasing amounts of potassium iodide to different serum specimens were investigated by analysis of covariance. Data management and computations were performed with the statistical software package STATISTICA/w from Statsoft (Tulsa, OK).

RESULTS

Application and Detection Limit of HPLC

Typical chromatograms obtained by HPLC are shown in Fig.1 for two concentrations of calibrator representing the highest and lowest points of the calibration curve and for a serum sample containing 0.14μmol/L iodide. The retention time of iodide is 5.3 min; the total analysis time for one sample is 7.5

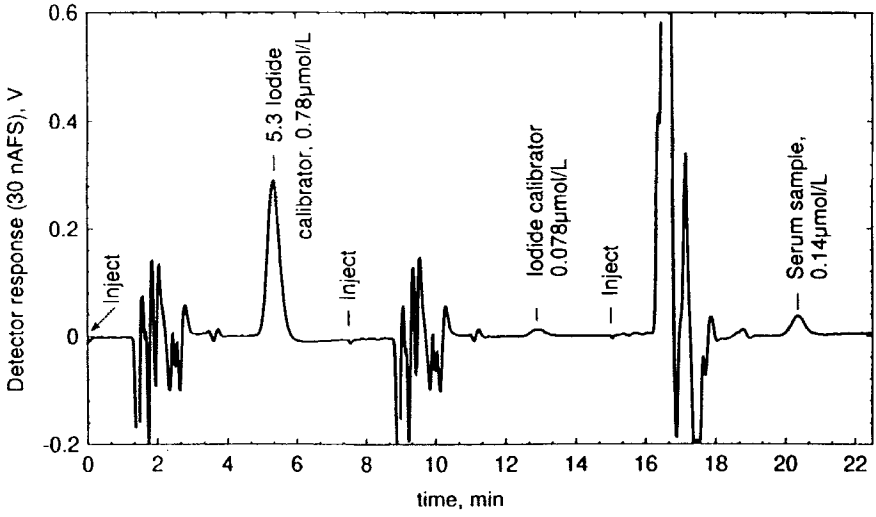

Figure 1. Chromatograms of calibrators containing 0.78 and 0.078 µmol/L potassium iodide and of a serum sample with 0.14 µmol/L inorganic iodide. Electrochemical detection was with 0.6 V integrator sensitivity corresponding to 30 nA full-scale (nAFS) detector sensitivity

min. The detector response shows a quadratic relationship with the iodide concentrations in the lower range between 0.078 and 0.78µmol/L (Fig.2). According to concentration of the ultrafiltrate, the usual detection limit of the HPLC assay of 0.04 µmol/L[17,18] based on a signal to noise ratio of 3, could be lowered by a factor of ten to 0.004 µmol/L.

Precision

The within and between-run precision (CV) for various serum iodide concentrations are given inTable 1. The intraassay-CV of each serum sample is based on 10 measurements within one run, the interassay-CVs were calculated from 10 determinations of the serum specimens over a period of 4 weeks.

Recovery of Iodide During Sample Preparation

To test the recovery of iodide during preparation of samples 2mL aliquots of 12 different serum specimens each spiked with 1kBq of ^{123}I were placed

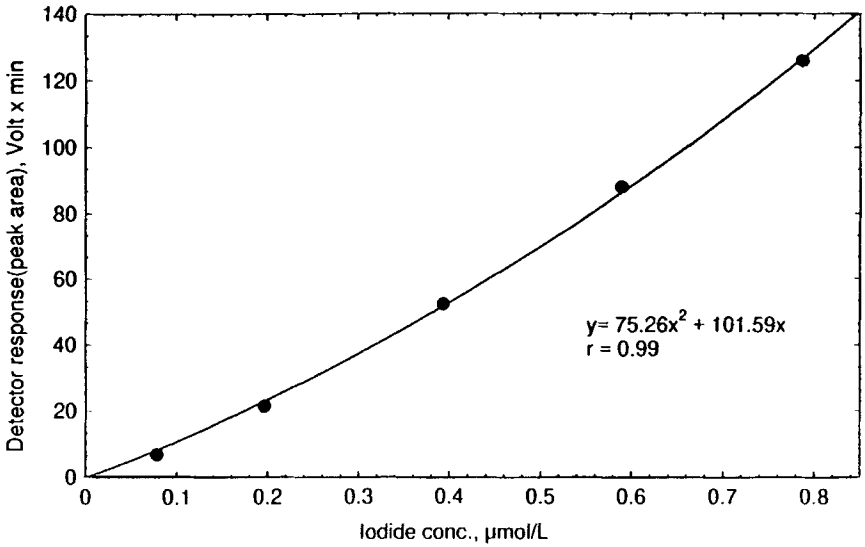

Figure 2. The calibration curve based on aqueous solutions of potassium iodide shows a quadratic relationship with the iodide concentrations in the lower range

onto filter cups and ultrafiltrated as described earlier. The radioactivity of the serum samples and of the ultrafiltrates was measured in a well-type counter. The calculated recovery was 90.8% ± 4.6%. 1200µL aliquots of the same radioactive ultrafiltrates were then concentrated in a Speed-Vac-concentrator by a factor of 10. The radioactivity of the ultrafiltrates before and after concentration was determined in the well-type counter, yielding a recovery of 102.2% ± 5.6%. The loss of iodide during ultrafiltration and concentration is, therefore, very low and negligible.

Recovery of Iodide in Serum

To detect possible interference effects by unknown compounds in serum, we supplemented four serum samples with iodide concentrations of 17 to 155 nmol/L before preparation with increasing amounts of potassium iodide and analyzed. The slope between the amount of iodide measured and the amount added was calculated for each sample. The results and the corresponding regression lines are shown in Fig.3.

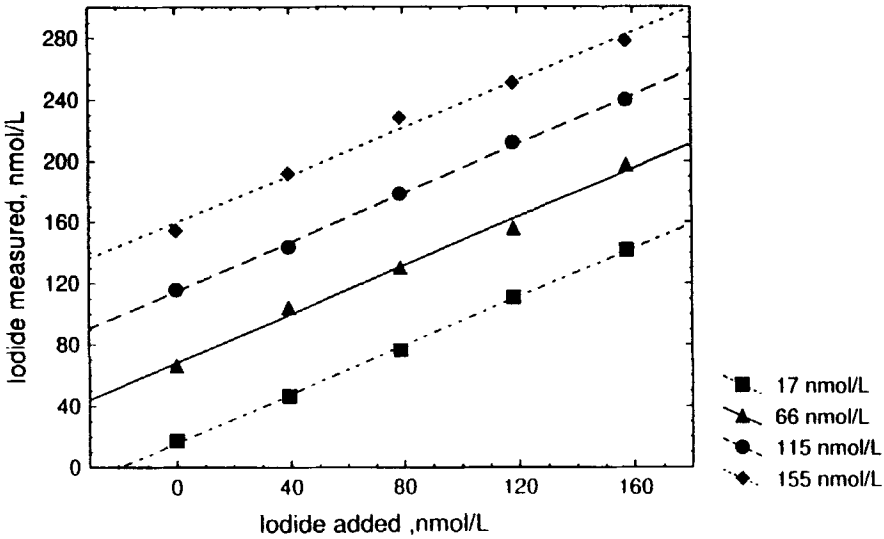

Figure 3. Recovery of iodide added to four different serum samples before preparation. Each point represents the mean of two experiments

There was no significant difference between slopes (P = 0.41). The mean slope was 0.80, suggesting an absence of interference effects and indicating that recovery of iodide was almost complete, which is in agreement with the calculated average recovery of 89.3%.

Comparison with the Reference Method

For comparison, the iodide content of 26 serum samples was determined by both HPLC and the reference method based on the isotope dilution principle. The calculated regression coefficient is 0.91 and the intercept does not differ significantly from 0 (Fig.4).

SII Levels in Iodine Deficiency

To determine the range of SII levels typical of an iodine deficient area, sera from 27 euthyroid children with low urinary iodine excretion (range: 14.5 -126.1 μmol/molCreatinine, median: 44 μmol/molCreatinine) were measured by HPLC. The results are given in Table 2 and are well comparable with those published in the literature for different iodine deficient areas (see Table 2).

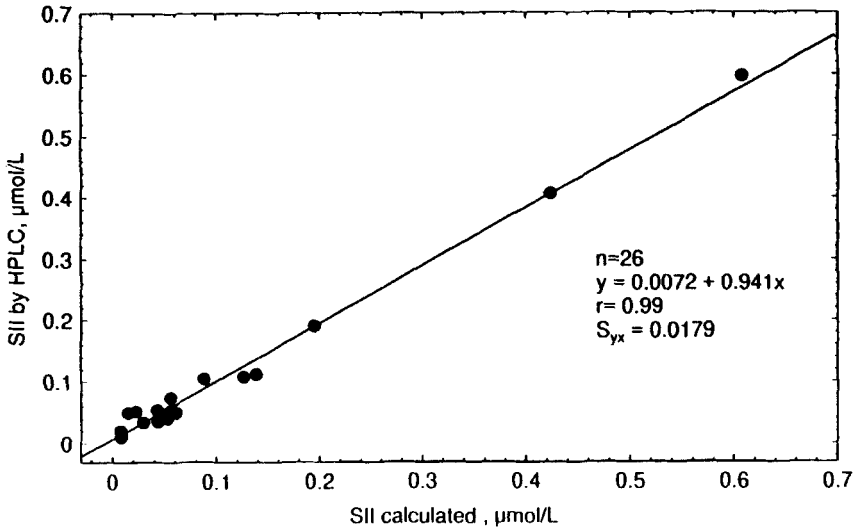

Figure 4. Comparison of SII concentrations in sera from 26 patients measured by HPLC with calculated SII values obtained from a tracer (^{123}I) method based on the isotope dilution principle.

Table 2

Levels of Serum Inorganic Iodide in Iodine Deficiency: Comparison of Own Data with those Published in the Literature for Euthyroid Subjects

Author	Ref.	n	Mean (µmol/L)	Range (µmol/L)
Koutras	13	13	0.020	0.008 - 0.080
Mantzos	21	10	0.016	0.006 - 0.031
Postmes	16	27	0.021	0.008 - 0.041
this study		27	0.023	0.010 - 0.036

DISCUSSION

In contrast to the measurement of urinary iodine, comparatively little attention has been given to the determination of serum inorganic iodide, possibly because of the technical difficulties of directly measuring SII, since very small quantities are involved. Especially in iodine deficient areas, levels

of SII significantly below 0.04μmol/L are usually found.[13,19,20] Furthermore, direct methods for the chemical determination of SII[16,21,22] use the colorimetric ceric-arsenic assays, which are based on the catalytic effect of iodide in the Sandell and Kolthoff reaction.[23,24] These methods, however, are subject to various potential sources of error, mainly due to the fact, that even slight contaminations from the PBI (protein bound iodine) fraction or from free thyroid hormones may greatly increase the SII value. In addition, the concentration of iodine in the reagents used for the separation and direct determination of iodine is usually several times higher than that of SII.[21] For that reason, most investigators used indirect methods for the estimation of SII, based on the isotope dilution principle and the determination of iodine in urine[10,13,15,19,25-28] or saliva.[20,29-32] SII has been determined, satisfactorily by applying these methods, but they are very cumbersome and associated with the administration of radioactive tracers. They exhibit additional disadvantages as Wayne[33] and Fitting[34] pointed out and therefore a direct method with the required accuracy, precision and sensitivity for measuring SII would be highly desirable.

As recently shown,[17] an alternative approach for determining iodide in biological fluids is afforded by paired-ion, reversed phase HPLC with electrochemical detection. This technique offers several advantages over the above-mentioned direct chemical methods. Unlike the chemical assays, which are sensitive to any iodine containing compound, the HPLC method showed almost complete absence of interfering substances due to the electrochemical detection in combination with the chromatographic technique.[17] The HPLC, therefore, measures selectively unbound iodide and its specificity is not impaired by contaminations from organically bound iodine.

Hurst et al.[35] described a HPLC method using electrochemical detection for the determination of iodide in serum, whereas Buchberger[14] measured SII by ion chromatography with post-column reaction detection. The least iodide concentrations detectable by the HPLC methods have been reported to range from 0.03 to 0.06 μmol/L,[17,18,35] the detection limit of the post-column reaction was 0.008μmol/L.[14] Compared to a normal range of about 0.004 to 0.04 μmol/L usually found for SII values in iodine deficient areas,[13,16,19-21] the chromatographic techniques are also too insensitive to detect such minute concentrations.

In our present study, we describe a relatively simple automated method for the direct determination of inorganic iodide in serum using paired-ion, reversed phase HPLC and ultrafiltration for removal of proteins. The required sensitivity was achieved by concentrating the iodide content of the ultrafiltrates with a Speed-Vac-concentrator. The recovery of iodide during preparation of

samples and during concentration of the ultrafiltrates was 90.8% and 102.2%, respectively. Therefore, the loss of iodide by ultrafiltration and concentration is very low. The data concerning accuracy and precision (s.Table 1) clearly document that the proposed method is an accurate, precise and effective alternative to the current procedures for determining serum inorganic iodide. Due to the relatively short retention time of iodide (5.3min) the total analysis time for one sample is 7.5min which gives a turnaround rate of eight samples per hour.

The present study compares, for the first time, data from serum samples measured for free inorganic iodide by both HPLC and a reference method based on the isotope dilution principle. There is almost complete agreement between the amount of inorganic iodide in the serum samples as measured by HPLC and the amount calculated according to the isotope dilution principle.

The SII values determined by HPLC, however, are slightly lower than the calculated ones (see Fig.4). These calculations are based on the assumption that the specific activity of serum inorganic iodide equals the specific activity of total urinary iodine. There is evidence, however, that the kidneys excrete into urine not only inorganic serum iodide but also organic iodine compounds in small amounts.[36-40] Furthermore, some of the inorganic iodide in the urine is derived from thyroidal hormones and from other organic iodine compounds metabolized and deiodinated during their passage through the kidneys.[38,40-42] Consequently, the specific activity of neither total urinary iodine nor inorganic urinary iodide equals exactly the specific activity of serum inorganic iodide. The calculated values of SII are, therefore, slightly higher than the values determined by HPLC or by saliva/plasma methods.[20,43]

Due to variations in the iodine intake values of SII reported by various authors differ widely. However, in iodine deficiency our results are well comparable with those published in the literature (s.Table 2), with a mean SII concentration of about 0.02 μmol/L. In contrast to this very low SII level, Hurst et al.[35] from Pennsylvania in the U.S., measured SII concentrations in 6 euthyroid subjects. As expected, according to the adequate iodine supply in the U.S., the mean value was significantly higher (0.70 μmol/L) than the mean SII concentration in iodine deficient areas.

In conclusion, the present study clearly demonstrates that paired-ion reversed phase HPLC with electrochemical detection, is an accurate and precise method for determination of SII. Comparison with a reference method based on the isotope dilution principle shows nearly complete agreement.

In contrast to these indirect methods, which are very cumbersome and associated with the administration of radioactive tracers to patients, or compared with direct chemical methods being subject to various potential sources of error, the HPLC assay is a relatively simple automated procedure for the direct determination of SII.

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