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Urine iodide determination by ion-pair reversed-phase high performance liquid chromatography and pulsed amperometric detection

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ABSTRACT

A sensitive and specific ion-pair reversed-phase high performance liquid chromatography (HPLC) method for urinary iodine analysis is described. This method is based on pulsed amperometric detection (PAD) using a silver working electrode (HPLC–PAD), which improves peak shape, electrode stability as well as linearity and reproducibility. A two-step extraction process consisting of solid phase extraction (SPE) and liquid–liquid extraction with dichloromethane was added in order to improve sample purification which is essential with the use of PAD. Treated samples were eluted on a C18 column, using a phosphate buffer containing ion-pairing reagent tetrabutylammonium and 5% MeOH. The calibration standard curves were linear up to 500 μg/L and within-run and between-run coefficients of variation (CVs) were $<6\%$ with the quantification limit fixed at 6 μg/L. Accuracy, expressed as recovery, ranged from 94% to 104%. Comparison with the Technicon AutoAnalyzer acid digestion (AA) method resulted in a high correlation ($r=0.9916$). Due to a low quantification limit and high sample throughput, the proposed technique appears suitable for both epidemiological and clinical follow-up studies.

1. Introduction

Iodine is an essential micronutrient utilized by the thyroid gland for the biosynthesis of thyroxin (T4) and triiodothyronine (T3). These hormones play a significant role in mental development, growth and basic metabolism. Iodine deficiency may lead to severe brain development delay. Contrarily, excessive iodine intake may cause goiter, hypothyroidism or hyperthyroidism [1]. Given that iodine deficiency is still endemic in many parts of the world [2], a reliable method is needed to evaluate dietary iodide intake and assess iodine status on a population-based level. As urinary iodine concentration reflects current dietary iodide intake, this parameter has been used as a marker of iodine status in population studies [3].

Several methods for measuring iodine are currently available, such as spectrophotometric methods based on the catalytic effect of iodide on the oxidation of As (III) by Ce (IV) (Sandell–Kolthoff reaction) [4–6], inductively coupled plasma mass spectrometry (ICP-MS) [7,8], neutron activation analysis (NAA) [9–11], and introduced more recently, electrospray ionization tandem mass spectrometry (ESI–MS–MS) [12]. However, most applied analytical methodologies are time-consuming and expensive, with the exception of capillary electrophoresis (CE) with direct UV detection [13].

Ion chromatography coupled with electrochemical detection, especially ion-pair reversed-phase high performance liquid chromatography (HPLC) provides an alternative to the above mentioned approaches, offering several advantages over standard ion chromatography [14–20]. First, there is no need to purchase expensive, special purpose ion-exchange columns and in addition, investment in extra-chromatographic instrumentation is seldom necessary, as separations can typically be performed with conventional HPLC systems. The electrochemical detection is commonly conducted using a silver electrode due to its high selectivity and very low detection potential [21,22]. However, when iodide comes in contact with the anodically poised Ag electrode, a current will flow with the concomitant precipitation of silver iodide on the electrode surface, leading over time to chromatographic postpeak distorsion, poor reproducibility, reduced linearity and signal drifts [22]. Recently, pulsed amperometric detection (PAD) using a silver electrode has been proposed in order to electrochemically clean the electrode surface, thereby improving the reproducibility of electrode responses [23–27]. The application of PAD to biological samples such as urine [26] and serum [27] was reported in the literature. However, applying the coupled ion-pair HPLC–PAD to biological samples has not yet been described, PAD being often coupled with anion exchange chromatography instead of ion-pair HPLC.

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We propose a simple, selective, and sensitive ion-pair HPLC method coupled with PAD using a conventional silver working electrode for routine urinary iodide measurements. This method is devoid of the aforementioned limitations. Our results were compared to those obtained by means of the automated Sandell–Kolthoff spectrophotometric method (Technicon AutoAnalyzer II) (AA) [4], which has been used for over 25 years in our laboratory for iodine status surveys in Belgium [2,28]. This method has been periodically subjected to routine external quality control.

2. Materials and methods

2.1. Chemicals and reagents

HPLC–PAD: Analytical-grade disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4 \cdot 12H_2O$) was obtained from VWR (Leuven, Belgium), while EDTANa_2H_2 (Titriplex III), di-*n*-butylamine, dichloromethane and ion-pairing reagent tetrabutylammonium phosphate (TBAP) were purchased from Sigma-Aldrich (Steinheim, Germany), with methanol and analytical-grade potassium iodide being obtained from Merck (Darmstadt, Germany). To investigate potential interferences with other ions, analytical-grade NaCl, NaBr, NaF, $Na_2S_2O_3$, $NaNO_2$, $NaNO_3$, KSCN and disodium oxalate were purchased from Sigma-Aldrich (Steinheim, Germany), with KIO_3 being obtained from Merck (Darmstadt, Germany).

All solutions were prepared using deionised water 18 MΩ cm from a resin Aqualab system (VWR, Leuven, Belgium).

2.2. Chromatographic system

The HPLC system (Shimadzu, Kyoto, Japan) consisted of the following components which were connected in series: dual-plunger parallel-flow solvent delivery module LC-20AD, on-line degasser DGU-20A5, auto-sampler SIL-20A, oven CTO-20AC and system controller CBM-20A. The signal was recorded with an electrochemical detector including an Ag/AgCl reference electrode, a 50 μm gasket defining an analytical cell volume of 2.5 μL, and a silver working electrode (Antec Leyden, Netherlands). The system controller and the detector were connected on-line to a Dell computer operating with the LC Solution software from Shimadzu (Kyoto, Japan). The chromatographic column was an X Terra® MS C18 reversed phase column, 3.9×150 mm^2, 9 nm, 5 μm (Waters Corporation, Milford, MA, USA) which was kept at 35 °C in the column heater.

The silver working electrode was treated as necessary with a polishing cloth and diamond slurry and then rinsed with deionised water.

The mobile phase consisted of water–methanol (95:5, v/v), pH 6.8 containing 2.5 mmol/L $Na_2HPO_4 \cdot 12H_2O$, 0.5 mmol/L EDTA-Na_2H_2, 2.5 mmol/L TBAP and 3 mmol/L di-*n*-butylamine.

2.3. Electrochemical detection and optimization

The electrochemical reaction occurred in a three-electrode analytical system where the potential of the working electrode (Ag) was measured and compared to that of a stable reference electrode (Ag/AgCl) through which no current flowed. A power supply maintained the working electrode at a potential of 0.1 V in comparison with the reference electrode potential.

The following tri-potential waveform was applied to the silver-working electrode vs. the Ag/AgCl reference electrode: $E_1 = -0.15$ V (t_1=0–1.9 s, t_d=700 ms), $E_2 = -1.15$ V (t_2=1.9–1.97 s) and $E_3 = -0.3$ V (t_3=1.97–2 s). This potential waveform was characterized by a detection potential of −0.15 V for 1.9 s (t_1) with current integrated between 1.2 and 1.9 s (time interval t_d), where the time difference of 0–1.2 s was the electrode stabilization time. Then, a cleaning pulse of −1.15 V during the time interval t_2, when Ag^+ in AgI precipitate was reduced to Ag^0, facilitated the removal of the AgI layer from the electrode surface. Finally, a rest potential of −0.3 V was applied for 0.03 s (t_3) before commencing a new cycle. The slow waveform time of 2 s was found to be necessary in order to completely stabilize the electrode during use.

The full-scale integrator sensitivity was 1.0 V and the detector sensitivity was 50 nA.

To select the optimum operating potential for determining iodide in the selected mobile phase, we generated a current–voltage curve by repeatedly injecting a calibration solution (100 μg/L potassium iodide) at various potentials. Increasing the potential in 0.05 V increments resulted in no further increase in signal response at a working potential of −0.15 V. We therefore used this potential under routine conditions as it was shown to be the lowest potential in the plateau region of the current–voltage curve, along with minimized noise.

2.4. Iodide calibrators

Working solutions of 20 μg/L, 50 μg/L, 100 μg/L, 200 μg/L and 250 μg/L were prepared from 100 mg/L iodide solution containing 130.8 mg of potassium iodide in 1 L of deionised water.

2.5. Collection of urine samples

Urine samples were analyzed using both HPLC and AA, with the samples originating from 490 patients for whom urinary iodine measurements were requested by their treating physicians. The samples were kept frozen until assayed.

2.6. Analytical procedure

Three millilitres of each urine sample were poured onto C18 Sep-Pak (Waters) extraction columns which were rinsed prior to use with 5 mL methanol and 10 mL deionised water. While the first 2 mL of eluate were discarded, the third millilitre was collected. A semi-automated Vacelut® (Agilent Vacuum Products Division, Middelburg, Netherlands) low pressure manifold was used for elution.

Five hundred microliters of the eluate or calibrator were collected into a clean tube, with 500 μL of aqueous TBAP solution (0.1 M) added. The formed ion-pairs were extracted using 5 mL dichloromethane after mixing at 2–8 °C for 30 min. Following 5 min centrifugation at 3500*g*, 4 mL of the organic phase was withdrawn and evaporated to dryness at 40 °C in a water bath. The dry residue was then mixed with 500 μL mobile phase.

All separations were carried out at 1 mL/min flow rate using an isocratic mobile phase, with an injection volume of 30 μL and analysis temperature of 35 °C. Concentrations were calculated based on peak areas using the automatic integrator LC-Solution. The calibration curve was plotted by linear regression.

Samples extracts with iodide concentrations exceeding the linearity limit (500 μg/L) were diluted with the HPLC buffer and injected directly into the system.

After use, a methanol/water (30:70, v/v) wash solution was passed through the HPLC setup at 0.3 mL/min for 45 min for rinsing, with the solution being kept in the system.

2.7. Statistical analysis

For comparison purposes, iodine concentrations of 490 random samples were determined using both the HPLC and AA methods. The AA method for iodine concentration measurements

has been extensively described elsewhere [5,29]. The Pearson correlation coefficient, the Passing–Bablok plot and the Bland–Altman difference plot were used to assess the agreement between HPLC results and data using the Sandell–Kolthoff method [30]. Statistical analyses were performed using the MedCalc program.

3. Results and discussion

3.1. Detection performance evaluation

When using constant DC amperometric detection, a significant baseline disturbance was observed, accounted for silver iodide deposits dissolving slowly from the electrode surface [22] (Fig. 1A).

Under PAD conditions, the resulting chromatogram showed an almost symmetrical peak shape, indicating proper cleaning of the electrode surface (Fig. 1B).

Furthermore, a progressive decrease in the signal response was obtained with DC amperometric detection (Fig. 2). On the contrary, the PAD response exhibited higher stability with no signal lowering.

The silver electrode was stable for at least 1000 urine samples without any need of cleaning. However, we preventively cleaned the electrode, as described above, at the time of column exchange, after each 500 injections.

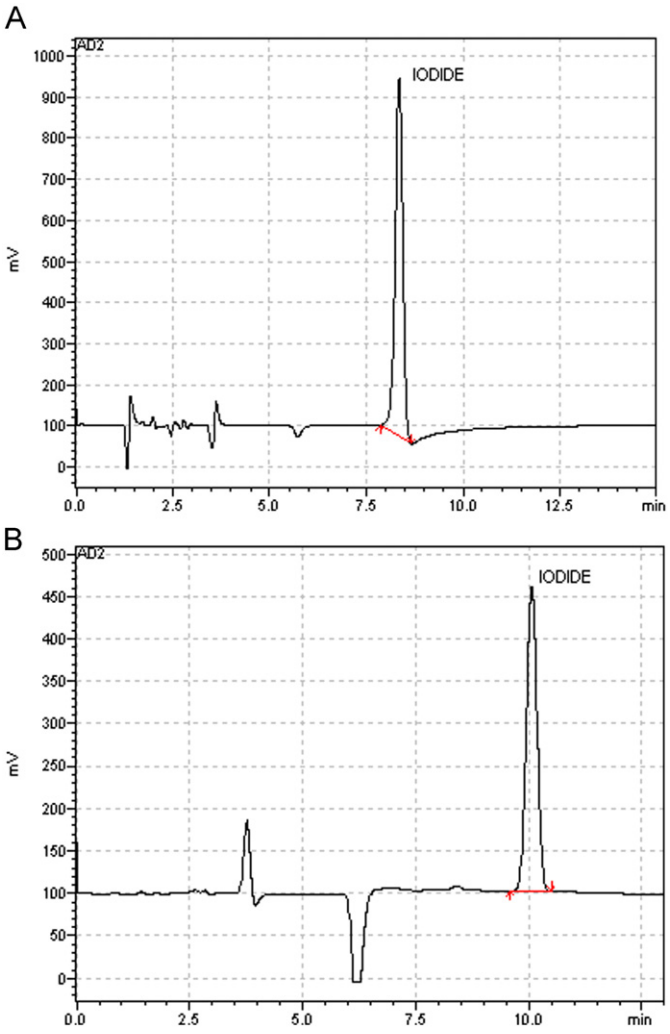

Fig. 1. DC amperometric detection (A) vs. pulsed amperometric detection (B) of 250 μg/L on an Ag electrode. A: −0.15 V vs. Ag/AgCl reference electrode was applied.

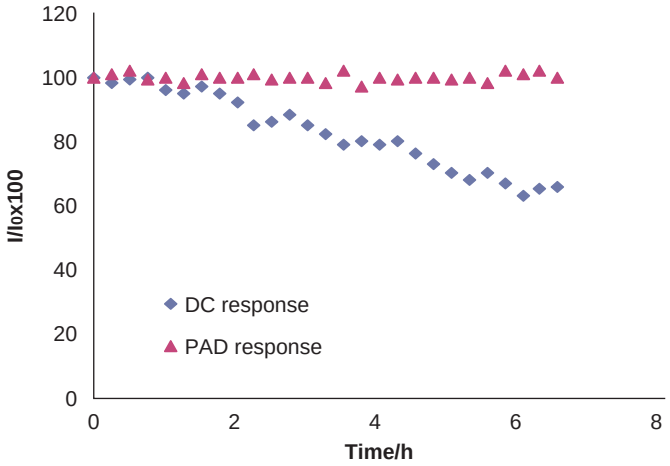

Fig. 2. DC amperometric response vs. PAD response of the silver electrode with a standard solution containing 250 μg/L iodide. The solution was injected for approximately six consecutive hours. A potential difference of −0.15 V vs. Ag/AgCl reference electrode was applied for DC amperometric detection.

3.2. Chromatograms

Typical chromatograms obtained with 250 μg/L standard and urine sample are shown in Figs. 1B and 3C. The retention time for iodide was 10.5 min which decreased gradually with the wearing of the column, approximating 8 min after 500 samples had been passed on the column. Each run took 13 min, with no late interference eluted at the time of iodide.

Under the preparation conditions described by Rendl et al. [14] on Waters Sep-Pak C18 extraction cartridges, chromatograms recorded with PAD revealed that filtrates were not sufficiently clean, with unidentified substances possibly co-eluting with iodide (peak X, Fig. 3A). After injecting several urine samples treated with dichloromethane alone without solid-phase extraction (SPE) on Sep-Pak C18, chromatograms were substantially cleaner with peak X of lower intensity, though still present (Fig. 3B). Therefore, we combined these two extraction steps and obtained a very low peak X excluding any possibility of interference (Fig. 3C).

Occasionally, very straight short spikes, such as the spike at 3.5 min as shown in Fig. 2B, were recorded in the chromatogram. This phenomenon was due to an overload of PAD detection when the recorded signal changed too quickly. With the two-step sample treatment, such spikes were seldom observed, and in our concentration range, this phenomenon never occurred at the iodide peak.

3.3. Analytical performance

3.3.1. Calibration curve and limit of linearity

The calibration line was adequately described by linear regression over a 20–250 μg/L concentration range and the calibration curve was linear up to 500 μg/L. One of the regression equations was $y=21{,}927x-309{,}327$. Calibration parameters were stable with regression coefficients being ≥ 0.994 in all cases. The mean absolute percentage deviation of standards was 5% or better, and in no case did an accepted standard deviate from nominal by more than 10%.

3.3.2. Precision and accuracy

Intra- and inter-assay CVs at different urine concentrations are shown in Table 1. Accuracy measured with spiked urines is given in Table 2, and accuracy measured with Ensuring the Quality of

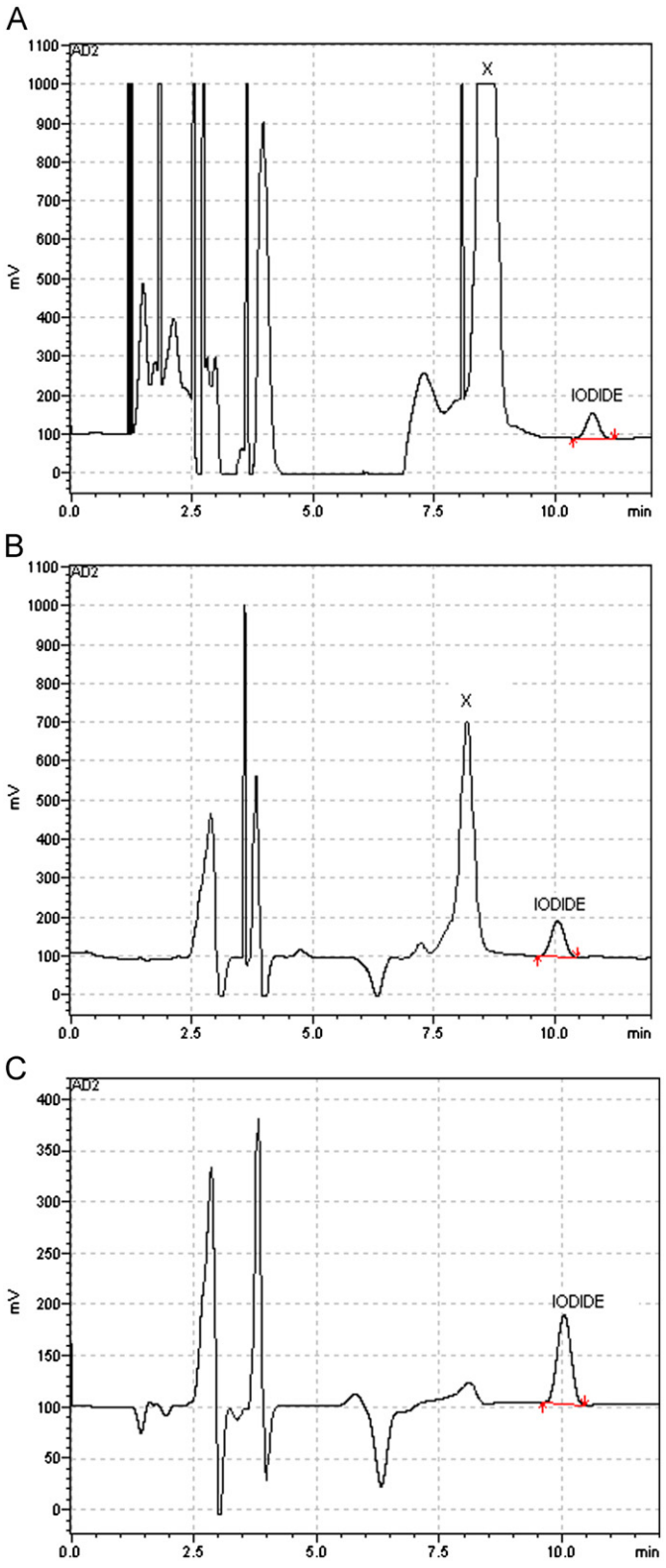

Fig. 3. Urine chromatographic profiles: (A) urine sample treated using Rendl's method [14]; (B) same sample treated only with dichloromethane, without SPE and Sep-Pak C18; and (C) same sample treated by the proposed method. The sample contained 110 μg/L of iodide.

Urinary Iodine Procedures (EQUIP) external control of CDC (Centers for Disease Control and Prevention, USA) in Table 3. Both accuracy and precisions were within acceptable criteria, precision being lower than 15% and mean accuracy within 85–115% [28].

Table 1
Assay precision.

n	Urinary iodide (μg/L)	CV intra-assay (%)	CV inter-assay (%)
20	61.0	2.6	3.7
20	150.0	3.1	5.8
20	374.0	0.9	1.2

Table 2
Accuracy assessment with spiked urines.

Target (μg/L)	Urinary iodide (μg/L)	Accuracy (%)
61	60.5	99.2
107	107	100
152	143	94.1
243	241	99.1
288	300	104
516	512	99.2

Table 3
Accuracy assessment with EQUIP external controls.

Sample	Target (μg/L)	HPLC result (μg/L)	Accuracy (%)
1	19.5 (13.7–25.4)	19.7	101
2	103.1 (82.5–123.7)	88	85
3	437 (309–572)	415	95
4	70.9 (53.2–86)	78	110
5	74.9 (56.2–93.6)	75.2	100
6	194 (136–252)	173	89

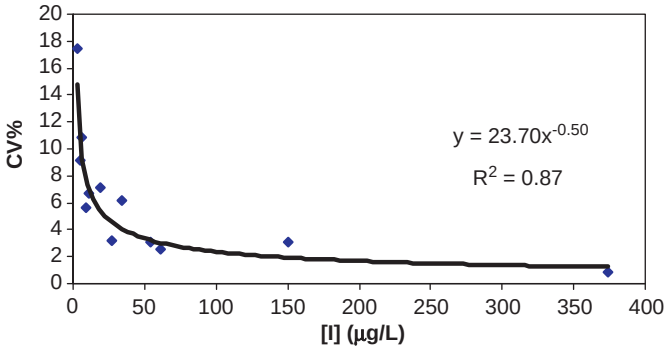

Fig. 4. Within-assay coefficient of variation profile of the method based on 10 single determinations on 12 urine specimens.

3.3.3. *Limit of quantification*

The limit of quantification, experimentally determined by analyzing successive diluted samples, was 6 μg/L. Mean precision for this limit value was found to be inferior to the maximum tolerable CV of 10% (Fig. 4), which is fully capable to detect severe iodine deficiency [3]. No matrix effect was observed with urine sample dilution, as a 90–112% recovery was obtained for serial 1:12 dilutions of urine sample at 61 μg/L, with deionised water.

3.3.4. *Study of interferences*

As shown in Fig. 5, chloride and bromide provided a response immediately after the void volume. A negative peak was detected after 5–6 min for all samples, probably corresponding to the reduction of dissolved oxygen. Thiosulfate was eluated at about 8 min, just prior to iodide. Thiocyanate exhibited a high peak at 50 μmol/L after 31 min. The other tested anions, notably fluoride, nitrite, nitrate, oxalate and iodate, showed no response at the applied potential and concentrations.

3.3.5. Comparison with Technicon AutoAnalyzer

There was a strong correlation between the two methods (Pearson's $r=0.99$). Urinary iodide concentrations measured using HPLC–PAD ranged from 10 to 4820 μg/L (median 82 μg/L), and total urinary iodine measured using AA from 13 to 4900 μg/L (median 91 μg/L). the Passing–Bablok comparison (Fig. 6) showed a good agreement with y-intercept, 4.01 (95% CI: 2.19–5.52); slope, 0.89 (95% CI: 0.87–0.90); $Sx/y=1.15$. However, the regression coefficient varied significantly from 1 and the results with the HPLC method were slightly inferior to those obtained when using AA. This was probably accounted for the improved specificity of HPLC that measured iodide, while AA measured total iodine. Agreement was also good in the lower concentration range, assessed using 270 samples with values ranging from 10 to 100 μg/L (Fig. 7). The Pearson correlation coefficient r was 0.91, and the mean absolute difference between the two methods over this concentration range was −3.1 μg/L (95% confidence interval: −22.1–15.9), with the Passing–Bablok regression $y=0.89x+4.07$.

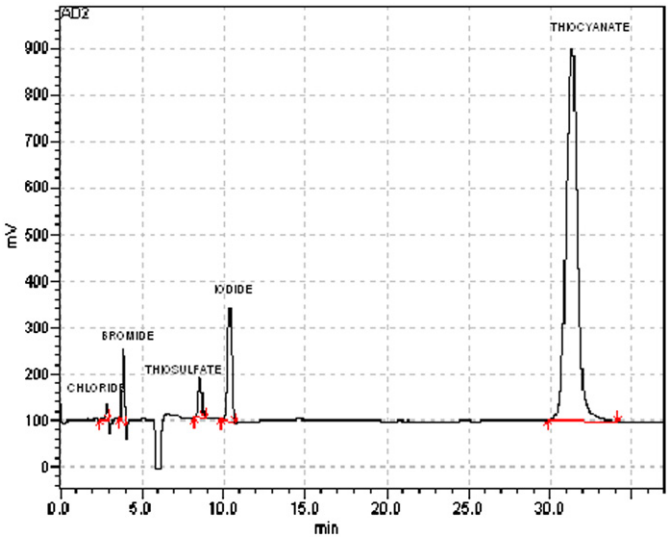

Fig. 5. Iodide (150 μg/L) in the presence of detected interfering anions: chloride (100 mmol/L), bromide (1 mmol/L), thiosulfate (1 mmol/L) and thiocyanate (50 μmol/L).

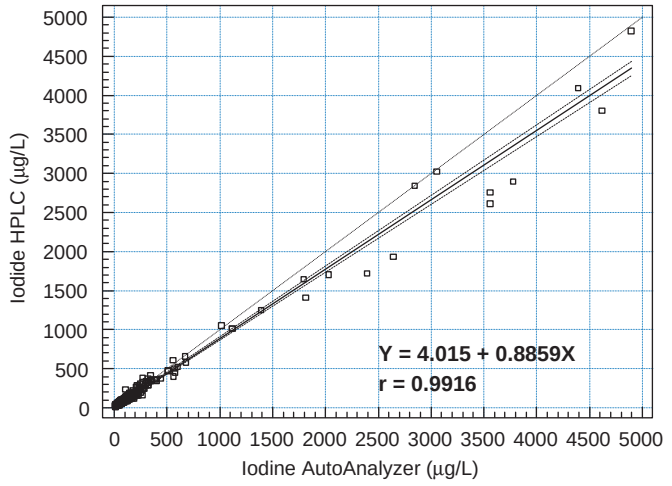

Fig. 6. The Passing–Bablok correlation of urinary iodine values measured using HPLC–PAD vs. Technicon AutoAnalyzer (Sandell–Kolthoff).

Analysis of urine samples from three patients who received water-soluble contrast media resulted in lower values obtained with our HPLC method (Table 4). According to the method's principles, these organic iodinated compounds are transformed into iodine and detected when using AA but they remain intact and are thus undetectable when using HPLC–PAD. Comparison of iodine results obtained with both methods for several usual iodinated contrast media such as iomeprol, ioxitalamate, iobitridol and amidotrizoate are shown in Table 5. For one of our patients, urinary iodine concentrations were assessed using both methods every day for 11 days, following an intravenous injection of 200 mL of Iobitridol containing 250×10^6 μg/L (Fig. 8). The amount of free iodide was found to be much lower than the total iodine amount, especially in the first day after intravenous injection. Nevertheless, urinary free inorganic iodide reached concentration in the range of 20,000–30,000 μg/L, thus about 100 times the recommended levels. In our view, these values may be explained primarily by the free iodide content of the contrast media and secondarily by the deionination of iodinated contrast media molecules occurring within the body [29]. As free non-organic iodide is known to be the metabolically important form of iodine [1], HPLC-determined iodide values are likely more relevant from a physiological point of view than total iodine values determined using AutoAnalyzer or other methods.

Table 4
Iodine amounts in urine following iodinated contrast media injection.

Sample	AA iodine (μg/L)	HPLC–PAD iodide (μg/L)
1	1100	223
2	1020	206
3	4260	1040

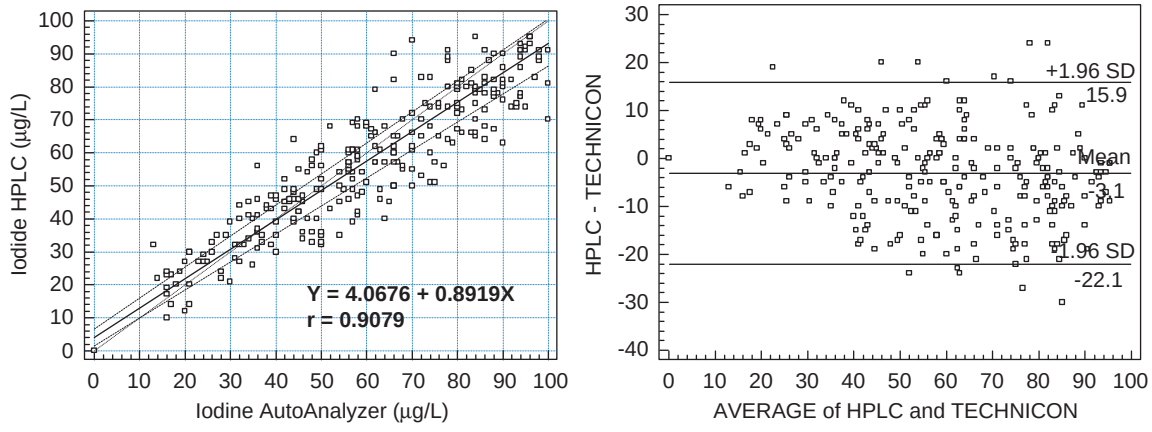

Fig. 7. The Passing–Bablok correlation and the Bland–Altman difference plot of urinary iodine values measured using HPLC–PAD vs. Technicon AutoAnalyzer (Sandell–Kolthoff) method over the 10–100 μg/L concentration range.

Table 5
Total iodine amount and free iodide determination in contrast media.

Iodinated contrast media	Dosage (μg/L)	Total iodine (AA) (μg/L)	Free iodide (HPLC) (μg/L)
Iomeprol (Iomeron®)	400×10^6	395×10^6	<6
Iobitridol (Xenetix®)	350×10^6	355×10^6	<6
Ioxitalamate (Telebrix®)	300×10^6	305×10^6	<6
Amidotrizoate (Urografine®)	146×10^6	160×10^6	<6

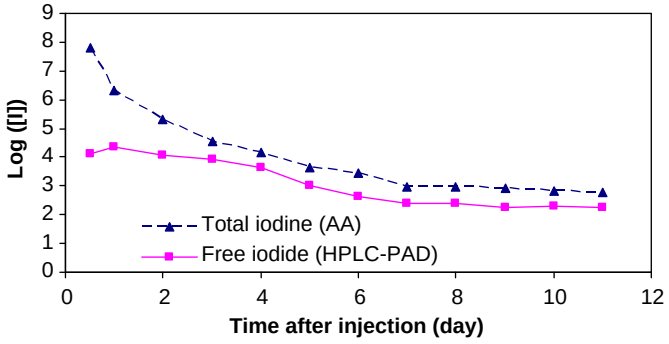

Fig. 8. Assessment of iodine/iodide excretion in one patient following iobitridol injection. [I] was expressed as μg iodine or iodide/L.

With respect to other sources of excess iodide, such as disinfectants, iodine containing amiodarone, seaweed and multivitamine preparations no differences were observed as these products are excreted in urine in the form of free iodide.

Compared to other methods, ion-pairing HPLC–PAD is relatively time-consuming, mainly because of the two-step pre-treatment. For one run of 50 samples, pre-treatment takes about 3 h, and HPLC analysis takes 13 min per sample. Furthermore, HPLC–PAD measures only unbound urinary iodide, which is, however, the physiologically active form, thus being more likely to be of clinical relevance in pathophysiological studies.

The HPLC method also avoids several disadvantages. Other methods that involve digestion procedures are sensitive to contamination. The Techicon AutoAnalyzer system demands special precautions against such hazards as potentially explosive perchloric acid. Although neutron activation analysis (NAA) [9–11] and mass spectrometry [7,8] meet sensitivity and accuracy requirements—NAA is considered the "gold standard" for measuring iodine [31]—the expensive instrumentation they require makes these methods unsuitable for routine practice.

4. Conclusion

In order to determine iodide concentrations in urine, a sensitive, selective, simple, precise and accurate method has been developed, which consists of two extraction steps, and a newly developed pulse sequence for amperometric detection using a silver-working electrode. The new pulse sequence was shown to improve peak shape as well as linearity and reproducibility, with no interferences being observed with other (pseudo) halides. With this method, the detector can be operated for months without surface cleaning or renewing. The results were almost identical to those obtained with the AA, when taking into account that HPLC measures unbound urinary iodide, whereas AutoAnalyzer, assesses the total iodine amount contained in a given sample. Urinary inorganic iodide measurements are more likely to be of clinical relevance in pathophysiologic studies as inorganic iodide has been shown to be the physiologically active fraction. In conclusion, the herein-described method appears to meet the criteria of an effective procedure for urinary iodine determination, notably rapid analysis, accuracy, interference freedom, low cost as well as the method's ability to detect even severe iodine deficiency, with urinary iodine levels <20 μg/L.

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