

Validation and Comparison of Two Calibration Methods for the Measurement of Stable Iodine in the Urinary Matrix by ICP-MS: Standard Addition vs. External Calibration

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Abstract

Background: In the context of a nuclear reactor accident, thyroid is the main target organ of radioactive iodines. To avoid as much as possible thyroid disorders or even cancer development, it is recommended to administer a single dose of potassium iodide to people at risk of exposure. Nevertheless, the Fukushima Dai-ichi disaster has pointed out many questions about the conditions of stable iodine prophylaxis implementation highlighting the need for reflection further revision of the actual “iodine doctrine”. Therefore, providing useful data is required notably through the implementation of animal experiments to strengthen current knowledge and to edit new recommendations. **Methods:** Urinary iodine constitutes a very good indicator to investigate the function of thyroid, its interpretation demands reliable analyses. Prior to perform animal experiments, two calibration methods were designed by our lab and compared together (standard addition and external calibration) to assess the urinary concentration of stable iodine in urine by ICP-MS. They were validated based on several key parameters especially linearity, accuracy and limits of detection (LOD) and quantification (LOQ). **Results:** The results were nicely satisfying. Indeed, both calibration methods have indicated very good coefficients of correlations, accuracies with low expanded relative uncertainties were obtained. The estimated LOD in the sample for standard addition method and external calibration were fully acceptable, 0.39 $\mu\text{g}\cdot\text{L}^{-1}$ and 0.35 $\mu\text{g}\cdot\text{L}^{-1}$, respectively. All performance criteria have been thus fulfilled successfully. The established methods were proven to be accurate, robust and sensitive. Once validated, both calibration methods were applied to rat urine samples and the results of z-score and Wilcoxon W test concluded that there

were no statistically significant differences between both methods.

Keywords

Stable Iodine, Urine, Standard Addition, External Calibration, Validation

1. Introduction

Among the halogen elements, iodine (I) is composed of thirty-seven isotopes among which only one is stable (^{127}I). Although its low quantity within the organism [1], I is a key micronutrient for the proper functioning of the human body. It is a major component in the synthesis of thyroid hormones especially tetraiodothyronine (thyroxine or T4) and triiodothyronine (T3) and thus is involved in many systems [2]. Among them, it plays a role on the intellectual development by participating in the brain maturation and also contributes to the regulation of many tissue functions (cardiac, muscle or digestive). Thus, the thyroid is a vital organ which may be the site of various disorders such as dys-thyroidism (hypothyroidism or hyperthyroidism) or cancerous endpoints meaningfully affecting the health status. In this regard, these major disturbances are greatly feared particularly following a nuclear reactor accident.

Indeed, when such accident occurs, a substantial fraction of releases is mainly composed of iodine isotopes, particularly ^{131}I with a physical half-life of eight days. Once ingested or inhaled, these radionuclides will bind on the thyroid gland where radioactive and non-radioactive iodine are not distinguished and may cause radiation injuries such as thyroid cancer or other thyroid diseases [3]. As an example, in the afterwards of the Chernobyl accident in 1986, a significant number of children at the time of the accident develops thyroid cancer primarily due to the exposure to radioiodines discharged from the plume [4]. As a protective measure against the development of childhood thyroid cancer, French health authorities as many other national organizations elsewhere had defined plans aiming to predistribute stable iodine tablets to people living in the vicinity of nuclear power plants and to organize larger geographical distribution in case of an nuclear accident occurrence. The objective of such countermeasure is to prevent the accumulation of radioactive iodine in the thyroid by administering a single dose of potassium iodide tablets (renewable once whether the evacuation of populations is impossible) to saturate the iodine binding sites [5]. To be effective, this drug must be ideally taken one hour before exposure [6]. However, this administration scheme does not consider the situation of repeated or prolonged releases as seen during the Fukushima Dai-ichi catastrophe. This point clearly represents a gap in the healthcare policies and therefore a major issue of concern for public health authorities [7] [8].

To interpret properly new experimental data gathered from animal testing that would serve to a significant revision of the iodine doctrine, it was necessary to design a reliable analysis method for the determination of iodine urinary ex-

cretion [9]. Various analytical methods have been already developed and are based particularly on the following techniques: instrumental neutron activation analysis [10], As-Ce catalytic spectrophotometry [11], ion selective electrodes [12], electrochemical detection [13], size chromatography exclusion [14], kinetic colorimetry [15] or else intracavity laser spectroscopic [16]. However, despite a higher cost compared to other techniques (due to the consumables including gas, input equipment maintenance, etc.), inductively coupled plasma mass spectrometry technique (ICP-MS) has become predominant in ultra-trace and multi-element analysis thanks to its attractive features like high selectivity, sensitivity, accuracy, low detection limits and its ability to discriminate the isotopes of the same element according to their mass [17]. Even so, the lower the half-life ($T_{1/2}$), the higher its specific activity should be. Therefore, the use of ICP-MS in radiotoxicology is mainly limited to stable and long-lived radionuclides ($T_{1/2} > 10$ years), such as ^{127}I or ^{129}I for iodine. For short-lived radionuclides (for instance, ^{123}I , ^{125}I or ^{131}I), radiometric methods will be preferred [18].

Iodine measurement by ICP-MS in various matrices (such as foodstuffs or biological fluids) has been already addressed in previous studies [19] [20] [21] [22]. For all these reasons, this analytical technique was naturally chosen for the measurement of iodine in the urinary matrix which was carried out by standard additions (referred as calibration 1) and external calibration methods (thereafter abbreviated as calibration 2). Moreover, recognizing that having access to facilities and authorizations required to manipulate radioactive iodine was challenging, we decided to manage our experiments with stable iodine (^{127}I) only. This article aims to compare with two types of calibration for ^{127}I determination, to report validation results and to present some data of urinary iodine in rodents (rats). This work will finally shed light on the most appropriate calibration method for urinary iodine determination not only in terms of accuracy but also for samples time preparation.

2. Materials and Methods

2.1. Instrumentation

Iodine determination was performed by ICP-MS using a quadrupole mass spectrometer “X Series II” (Thermo Fisher Scientific™, France). From a ASX-520 autosampler (Cetac Technologies, USA), the sample is injected and transported by specific tubings through the sample introduction system composed by a standard peristaltic pump and a Meinhardt nebulizer fitting into a classic spray chamber. The sampler and skimmer cones used were in nickel. The skimmer cone was of type Xt more suited to loaded matrices. For each experiment, all instrument parameters controlled from PlasmaLab software version 2.6.3.340 (2007) were optimized daily using a tuning iodide solution ($40 \mu\text{g}\cdot\text{L}^{-1}$) to obtain the highest sensitivity and to minimize possible interference effects (oxide levels and doubly charged ions). To get a general picture, hereafter values of ICP-MS acquisition settings that we could have: forward Rf power (1403 W), pole bias (-6.00 V), hexapole bias (-1.70 V), cool gas flow ($13.02 \text{ L}\cdot\text{min}^{-1}$), auxiliary gas

flow (0.80 L·min⁻¹) and nebulizer flow (0.76 L·min⁻¹).

Iodine was determined at $m/z = 127$ and tellurium (Te), as internal standard, at $m/z = 125$ (¹²⁵Te) in the pulse counting mode of data acquisition.

2.2. Chemical and Standard Solutions

Ultrapure water used throughout all experiments was obtained from a Milli-Q® Synergy 185 water purification system (Millipore, Saint-Quentin-en-Yvelines, France) with a resistivity about 18 MΩ. cm. Employed reagents were obtained from different suppliers and selected after careful checking of their low ¹²⁷I concentration. All necessary stock solutions were prepared the day of the experiment.

1) In this present work, 25% ammonia solution Suprapur® (MERCK MILLIPORE, Fontenay-sous-Bois, France) and 20% Normapur® (PROLABO, Fontenay-sous-Bois, France), of analytical-grade were used. To dilute the samples, we prepared 2% ammonia hydroxide solution (2% NH₄OH) by dissolving appropriate volumes of 25% ammonia solution Suprapur® in ultrapure water. In contrast, during measurements, the rinse solution used for ICP-MS between samples consisted of 5% ammonia hydroxide solution prepared from 20% ammonia solution Normapur®.

2) Regarding iodine oxidation state in solution which is a critical issue for iodine determination, to avoid iodide (I⁻) oxidation and then to stabilize these ions [23], we chose to add a strong reducing agent, sodium thiosulfate pentahydrate Suprapur® of analytical-grade (MERCK MILLIPORE, Fontenay-sous-Bois, France). A sodium thiosulfate stock solution was prepared at a concentration of 184 mg·L⁻¹ in 2% ammonia solution.

3) To correct any signal intensity variations, ¹²⁵Te was selected as internal standard because of its close physicochemical properties to iodine (particularly atomic mass and first ionization energy). ICP standard of Te (1000 mg·L⁻¹) in 2% - 3% nitric acid (MERCK MILLIPORE, Fontenay-sous-Bois, France) was purchased and this standard was diluted in 2% ammonia solution to reach a ¹²⁵Te concentration of 1.25 mg·L⁻¹ (or 17.68 mg·L⁻¹ in total Te).

4) For the calibration range of our two methods, from iodide certified standard solution in aqueous solution commercially available (1000 mg·L⁻¹) (VWR, Fontenay-sous-Bois, France) stored at 2°C - 8°C, the iodide calibration solution at 100 µg·L⁻¹ was prepared by performing two successive dilutions. For both methods, the calibration range included six points between 0.4 and 8 µg·L⁻¹ (0.4 ± 0.010, 1.0 ± 0.019, 2.0 ± 0.110, 4.0 ± 0.146, 6.0 ± 0.175, 8.0 ± 0.186).

2.3. Reference Materials

The validation of the methods has been carried out with certified reference materials (CRM, Seronorm™ Trace Elements Urine, ref. 210305 and 210705) obtained from Ingen, France. As no CRM rat urine was unfortunately commercially available to our knowledge, we used human urine CRM to perform the validation. These CRMs, stored at 2°C - 8°C, are lyophilized human-based control

materials produced from voluntary donors. As recommended, a volume of 5 ml of ultrapure water was added in the urine in order to reconstitute them. To confirm the applicability of our analytical methods, two levels of I^- certified concentration in urine are commercially available, $84 \pm 6 \mu\text{g}\cdot\text{L}^{-1}$ and $304 \pm 22 \mu\text{g}\cdot\text{L}^{-1}$. Furthermore, validation process was also performed at an iodide concentration of $42 \pm 2.95 \mu\text{g}\cdot\text{L}^{-1}$, from the CRM at $84 \mu\text{g}\cdot\text{L}^{-1}$ after a 2-fold dilution in ultrapure water. Before ICP-MS measurement, total dilution factors of 63 (for CRMs at 42 and $84 \mu\text{g}\cdot\text{L}^{-1}$) and 125 (for CRM at $304 \mu\text{g}\cdot\text{L}^{-1}$) were applied respectively.

2.4. Iodine Analysis Procedure

2.4.1. Calibration 1 (Standard Addition)

For this method, the first step involved the preparation of a pool composed of sodium thiosulfate ($184 \text{ mg}\cdot\text{L}^{-1}$), ^{125}Te and urine CRM. Then, the standard additions were prepared. For this, 1.2 mL of the pool was added in a 15-mL light sensitive centrifuge tube (VWR, Fontenay-sous-Bois, France) to the appropriate amounts of iodide solution to obtain 10 mL calibration solutions of concentrations between 0.4 and $8 \mu\text{g}\cdot\text{L}^{-1}$, the tubes were finally filled up to 10 mL with 2% ammonia solution. **Table 1** summarizes the standard additions preparation.

2.4.2. Calibration 2 (External Calibration)

For calibration range (**Table 2**), 100 mL volumetric flasks were used (VITLAB GmbH, Grossostheim, Germany) in which appropriate volumes of iodine standard solution ($100 \mu\text{g}\cdot\text{L}^{-1}$) were added to the pool to get a calibration range from 0 to $8 \mu\text{g}\cdot\text{L}^{-1}$. The calibration solutions contained also ^{125}Te and sodium thiosulfate solution ($18.4 \text{ mg}\cdot\text{L}^{-1}$).

The sodium thiosulfate solution ($18.4 \text{ mg}\cdot\text{L}^{-1}$) was prepared in the same conditions as calibration 1. Indeed, from the beginning to the end of the sample preparation, sodium thiosulfate solution underwent a 10-fold dilution.

For sample preparation, CRMs were diluted in 15-mL light sensitive centri-

Table 1. Standard additions preparation.

Concentration ($\mu\text{g}\cdot\text{L}^{-1}$)	0 (Blank)	0 (Blank)	0	0.4	1	2	4	6	8
Iodine $100 \mu\text{g}\cdot\text{L}^{-1}$ (en μL)	0	0	0	40	100	200	400	600	800
2% NH_4OH (mL)		8.8		8.76	8.7	8.6	8.4	8.2	8.0
Pool (mL)					1.2				

Table 2. External calibration standard solutions preparation.

Concentration ($\mu\text{g}\cdot\text{L}^{-1}$)	0	0.4	1	2	4	6	8
Iodine $100 \mu\text{g}\cdot\text{L}^{-1}$ (mL)	0	0.4	1	2	4	6	8
Sodium thiosulfate solution ($18.4 \text{ mg}\cdot\text{L}^{-1}$) in 2% NH_4OH				up to 100 mL			
Tellure 125 ($1.25 \text{ mg}\cdot\text{L}^{-1}$)				500 μL			

fuge tube by using the adequate dilution factor (63 or 125 depending on the iodine concentration) with ^{125}Te , and sodium thiosulfate solution ($18.4 \text{ mg}\cdot\text{L}^{-1}$) in the same proportions as for the calibration range.

Both established analytical methods required 300 seconds for sample analysis and 300 seconds for rinsing the equipment; such precautions were taken to maintain the lowest possible iodine signal (around 400 cps). For statistical purposes, each sample was prepared three times, and therefore in total thirty replicates of each sample were obtained. Finally, for calibration 2, the reagent blank signal was subtracted from each sample signal.

2.5. Methods Validation Process

The validation is an essential process towards the implementation of a new analytical method. This represents a quality assurance pledge and thus allows the laboratory to assess data reliability for further interpretation. In the present work, the validation consisted of evaluating the relevant performance criteria. To this end, the laboratory relied on several regulatory standards [24], offering methodological principles and recommendations. The lab's challenges were as follows: 1) to investigate the calibration function (linearity), 2) to study the accuracy of the method, 3) to assess the limit of detection (LOD) and the limit of quantification (LOQ) and to check the previously measured LOQ, 4) to determine the specificity by characterizing the yields and 5) to avoid any cross-contamination. All the previously mentioned criteria studied for both proposed methods have required several assays and then were evaluated using statistical tests as described below.

2.5.1. Linearity

For the linearity study, six and seven sets of experiments for calibration 2 and calibration 1 methods respectively, were performed on six calibration standard solutions ($0.4, 1, 2, 4, 6$ and $8 \text{ }\mu\text{g}\cdot\text{L}^{-1}$) and each experiment was repeated three times. To validate the linearity range, a statistical fit test (Fisher) was applied. It compared the observed model error and the observed experimental error using the analysis of variance, considering a normal distribution of the data.

2.5.2. The accuracy of the Method

The measurement accuracy is the closeness of agreement between the measured quantity values to that quantity's true value. The study of accuracy implies the evaluation of intermediate precision and measurement trueness expressed in terms of bias with respect to a reference value. The intermediate precision which represents the intra-laboratory reproducibility consists of analyzing a same sample under different conditions by varying for example at least one of the factors such as the operator or the time. Finally, the accuracy interpretation was based on the Fisher F test conclusion that addresses also the experimental condition changes.

To assess the accuracy of the method, the validation plan for calibration 2 consisted of six sets of experiments on the six calibration standards ($0.4, 1, 2, 4, 6$

and 8 $\mu\text{g}\cdot\text{L}^{-1}$) and three experiments per set per level, resulting to a total of one hundred and eight values. For calibration 1, seven sets of experiments were taken into account with six levels of concentration and three experiments per set per level resulting to a total of one hundred and twenty six values.

2.5.3. Limit of Detection and Limit of Quantification

LOD is the lowest concentration of the analyte to be detected but not quantified, and LOQ is the lowest concentration of the analyte to be quantified, under defined experimental conditions. They were determined respectively as three times and ten times the standard deviation of the concentration in ten reagent blank aliquots (with 30 replicates per sample). Then, the results were multiplied by the appropriate dilution factor to obtain LOD and LOQ in the sample matrix.

Once LOQ defined, the objective was to verify the accuracy of the measured LOQ within an acceptable maximum deviation of 60% by checking Inequalities (1) and (2):

$$\overline{\overline{Z}}_{LOQ} - 2 \times S_{LOQ} \geq LOQ - 60\% \times LOQ \quad (1)$$

$$\overline{\overline{Z}}_{LOQ} + 2 \times S_{LOQ} \leq LOQ + 60\% \times LOQ \quad (2)$$

The parameters “ $\overline{\overline{Z}}_{LOQ}$ ” and “ S_{LOQ} ” correspond to the overall mean and the intermediate precision standard deviation, respectively. The value of 60% was defined by convention [24].

To test the LOQ, the reagent blanks were spiked with iodine at a concentration equal to the LOQ defined above. Five sets were considered in intermediate precision conditions (for calibration 1, four experiments were performed) with four repetitions per set.

2.5.4. Specificity

The specificity of a method is its ability to measure only the analyte of interest. The analysis of this criterion allows knowing whether the measured response was disturbed by physico-chemical species other than the analyte of interest. Indeed, these constituents may be an important source of systematic errors.

To verify the absence of interfering substances, seven and five sets of experiments (each experiment was repeated three times) for calibration 1 and 2, respectively were considered. According to the regulatory standards [24], it is advisable to choose additions representing 20% and 80% of the highest calibration concentration. For calibration 2, iodine considered concentrations were 1.6 and 6.4 $\mu\text{g}\cdot\text{L}^{-1}$. For calibration 1, due to technical problems, three additions were envisaged, 0.67, 1.33 and 2.43 $\mu\text{g}\cdot\text{L}^{-1}$ corresponding to about 8%, 17% and 30% of the highest calibration concentration.

Then, for each experiment, the yield (or ratio between the observed and expected concentration of the addition) was calculated as well as the yield mean. Finally, the calculated recovery rates (referring to the overall yields, for a given addition) were considered acceptable within 90% - 110% of expected spike value.

2.5.5. Study of Possible Cross-Contamination

To identify a possible cross-contamination, for both calibration methods, an experiment was conducted by analyzing three samples at $8 \mu\text{g}\cdot\text{L}^{-1}$ and then three samples at $0.4 \mu\text{g}\cdot\text{L}^{-1}$. Afterwards, a contamination value was calculated according to the following Equation (3):

$$\text{Contamination}(\%) = \frac{(L_1 - L_3)}{(m_H)} \times 100 \quad (3)$$

with L_1 , L_3 and m_H referring to the concentration of the first measured sample at $0.4 \mu\text{g}\cdot\text{L}^{-1}$, the concentration of the third measured sample at $0.4 \mu\text{g}\cdot\text{L}^{-1}$ and the mean value of the highest calibration standard, respectively.

Finally, the contamination value was compared to a performance criteria set at 5%.

2.5.6. Urinary Iodine Estimation in Rats

Animal experiments were performed in 3-month-old male Wistar rats purchased from Charles River Laboratories (France) and were handled according to the French Legislation and the European Directives regarding the care and use of laboratory animals. Experimental protocols were validated by the IRSN's Ethics Committee. Rats were caged in metabolic cages for a 2-day acclimation period prior the experiment. They had free access to drinking water and food (with granules A04 having iodine content of $0.3 \text{ mg}\cdot\text{kg}^{-1}$ from SAFE, Augy, France) before and throughout the experimental period. During experiment six rats with normal iodized diet were gavaged once with 1 mL of injection water at pH 7.4 (control rats) and six rats with normal iodized diet were gavaged once with 1 mL of $0.35 \text{ g}\cdot\text{L}^{-1}$ potassium iodide (French Army Central Pharmacy, Orléans, France) (treated rats). The 24-hour rat urines were collected at different times: for half of the control and treated rats, urine samples were collected 24 hours after gavage. For the other rats, urine collection was performed 48 hours after gavage. All urine samples were frozen at -18°C . Then, urine samples collected a few months earlier were analyzed in triplicate using both methods, after dilution in 2% ammonia solution (400-fold dilution for control rats with both methods and relating to treated rats; for urines samples collected 24 hours and 48 hours after gavage, factors dilution applied were 7000 and 700, respectively for both methods).

2.6. Statistical Analysis

The experimental data were analyzed using GraphPad software version 5.0.

Once all experiments were carried out, prior the evaluation of each criteria, the distribution of the measured data was examined by using Shapiro-Wilk W test. A Gaussian distribution was observed for all data at each concentration level. Relating to Wilcoxon W test employed for comparison of both methods on rat urine samples, it is considered that no statistically significant difference is observed between the results when $p > 0.05$.

3. Results and Discussion

3.1. Memory Effects

Because of its physico-chemical characteristics, iodine determination requires special precautions in order to prevent any losses and memory effects particularly on the instrument. The first issue concerned the material used for sample preparation which constituted a critical step. To avoid any sample pollution, the utilization of single use material was proved to be necessary.

The second question raised was about the sample dilution medium. It is well known that in acid media, iodine is less stable [25]. Moreover, with iodate (IO_3^-) or I^- form, highly volatile molecular iodine (I_2) is generated. To overcome this problem, it is recommended to dilute the samples in alkaline solution to ensure the iodine chemical stability [26]. For this purpose, three basic solutions were investigated: sodium hydroxide (NaOH), tetramethylammonium hydroxide (TMAH) and NH_4OH . The first was promptly abandoned because sodium ions cause a decrease of plasma temperature affecting iodine ionization. Sample dilution in NH_4OH was chosen as on one hand iodine background was 2.5 times lower than the one with TMAH and as on the other hand, after the run of a highly contaminated sample (approximately $40 \mu\text{g}\cdot\text{L}^{-1}$) the time requested to find a satisfactory background was faster with NH_4OH .

Finally, the cost of TMAH is not negligible representing an additional point to consider.

As we can notice, everything is done to preserve the stability of iodine during the analysis. Nevertheless, in order to verify the absence of any pollution in ICP-MS experiments, we regularly place a reagent blank (2% NH_4OH). In this way, we assure to return to an iodine background (approximately 400 cps).

3.2. Internal Standard

It is well known that urine constitutes a complex matrix due to the presence of organic and inorganic substances. That is why, the choice of the internal standard is a key element because its spike in the sample is intended to correct any signal drift and matrix effects. According to the literature, two standards are regularly employed in iodine measurement: iridium 193 (^{193}Ir) and tellurium 125 [27]. A first set of experiments with urine samples spiked with these both internal standards shows that ^{193}Ir was not stable over time under our experimental conditions. In addition, its mass and its potential first ionization do not make it as a good candidate. Regarding our two methods, we used the results with and without ^{125}Te correction. It turned out that for the standard addition method, ^{125}Te allows to efficiently correct the instrumental drift and thus to be closer to the expected values. For this calibration method (1), ^{125}Te correction has been thus applied to all samples signals.

Nevertheless, for calibration 2 (external calibration), the sample comparison of the signals obtained with and without ^{125}Te correction showed that the ^{125}Te correction was not appropriate. Along ICP-MS measurements, the signal at mass

^{125}Te fluctuated too widely in the standard solutions medium (sodium thiosulfate in 2% NH_4OH) compared to our matrix of interest (urine), signal drift and matrix effect could not be offset. In most cases, the relative biases calculated by taking into account ^{125}Te correction were farther the target value. Consequently, for calibration 2, we refrained to use ^{125}Te correction.

3.3. Validation Process

3.3.1. Linearity

Measured iodine concentrations are compared to expected ones in **Table 3** and **Table 4**. A very good agreement is observed since the ratio for each calibration point is close to 1. Concerning calibration 2, a slightly greater dispersion is noted at the highest concentrations and explains a lower coefficient of correlation value (0.9993) but still remains acceptable.

To verify the calibration curve linearity, the Fisher F test was implemented to test the hypothesis of non-validity of the linear range. The observed F value was compared with a F value from Fisher's table with a α error risk of 1% and with the degrees of freedom p and $p(n - 1)$ (p representing the number of standards and n the number of replicate per experiment).

The observed F values were 0.81 (with $F_{table} = 3.35$) and 0.45 (with $F_{table} = 3.47$) for calibration 1 and calibration 2 respectively. For both, the calculated F value was less than the F table value at the risk of 1%. Therefore, the model error was

Table 3. Calibration 1: ratios between expected values and measured values.

Standard addition method	Ratio (expected value/measured value)					
	Calibration point n°1	Calibration point n°2	Calibration point n°3	Calibration point n°4	Calibration point n°5	Calibration point n°6
Experiment 1	1.02	0.97	1.00	1.00	1.00	0.99
Experiment 2	1.01	1.01	1.00	1.00	0.99	1.01
Experiment 3	0.97	0.95	0.99	0.99	0.98	0.97
Experiment 4	1.00	1.00	1.01	1.01	1.01	1.00
Experiment 5	1.02	0.99	1.02	1.00	1.01	1.00
Experiment 6	0.98	1.00	1.01	1.02	1.02	1.00
Experiment 7	1.00	0.99	0.99	0.99	1.00	0.99

Table 4. Calibration 2: ratios between expected values and measured values.

External calibration method	Ratio (expected value/measured value)					
	Calibration point n°1	Calibration point n°2	Calibration point n°3	Calibration point n°4	Calibration point n°5	Calibration point n°6
Experiment 1	1.03	1.00	1.02	1.00	1.00	1.00
Experiment 2	1.04	1.03	1.02	1.03	1.02	1.03
Experiment 3	1.06	1.02	1.00	1.02	0.99	1.02
Experiment 4	0.97	0.98	0.98	0.97	0.98	1.00
Experiment 5	0.95	0.92	1.04	0.95	0.96	0.96
Experiment 6	1.00	1.01	0.99	0.99	1.00	1.01

negligible compared to the observed experimental error. The assumption of non-validity of linearity range was denied. Thus, the calibration curves are considered linear in the concentration range investigated.

3.3.2. Accuracy

Before assessing accuracy parameters, it is recommended to apply the Cochran C and the Grubbs G statistical tests to identify the potential presence of aberrant or suspicious data. The Cochran C test examines the homogeneity of variances while the Grubbs G test explores the homogeneity of means. These tests were employed for each standard of both methods. The results have shown that the calculated values were all below the critical values at risk 5% and 1%, meaning that no doubtful value was detected. The next step consisted of determining the precision by focusing on the coefficients of variation of repeatability (CV_r) and intermediate precision (CV_R) and the evaluation of the trueness through the bias (corresponding to the systematic error estimation).

The most relevant results are reported in **Table 5** and **Table 6**.

First of all, as shown in **Table 5** and **Table 6**, CV_r and CV_R are very satisfactory with values below 5% (excepted for one value for calibration 2) implying a closeness of the measurements around the mean and so reflecting methods of very good precision. Then, relating to the trueness, as noticed, according to Equation (4), relative biases calculated varied from -0.56% to 0.44% for calibration 1 and ranged from -1.37% to 0.58% for calibration 2.

$$Bias(\%) = \left(\frac{\bar{\bar{z}} - Ref}{Ref} \right) * 100 \quad (4)$$

with Ref being the target value and $\bar{\bar{z}}$ the mean of the means.

Bias interpretation is based on normalized deviation (ND) which is described by the Equation (5) below.

$$ND = \frac{|\bar{\bar{z}} - Ref|}{\sqrt{\left(\frac{S_R^2}{n} \right) + u_{Ref}^2}} \quad (5)$$

with S_R^2 referring to the intermediate precision variance, n corresponding to the number of experiments and u_{ref} relating to the uncertainty of the target value.

As illustrated in **Table 5** and **Table 6**, all biases are widely less than 2% suggesting that biases are considered statistically negligible. However, the accuracy study also requires verifying Inequalities (6) and (7), involving the upper/lower acceptance and tolerance limits.

$$Ref - MDA < \bar{\bar{z}} - 2 \times S_R \quad (6)$$

$$\bar{\bar{z}} + 2 \times S_R < Ref + MDA \quad (7)$$

with MDA meaning Maximum Deviance Acceptable.

The terms “Ref + MDA” and “Ref - MDA” refer to the upper and lower acceptance limits, respectively whereas “ $\bar{\bar{z}} + 2 \times S_R$ ” and “ $\bar{\bar{z}} - 2 \times S_R$ ” relate to the upper and lower tolerance limits, respectively.

These previously mentioned inequalities were calculated for each level of standard and we can observe that the tolerance intervals are comprised within the acceptability limits. In all cases, the accuracy was confirmed.

Nevertheless, to strengthen these conclusions, it is necessary to perform the Fisher F test which judges the influence of modified conditions (operator, time). The results obtained are tabulated in **Table 5** and **Table 6** and are all below F value from Fisher’s table at risk 5% (2.848 and 3.106 for calibration 1 and 2, respectively), confirming that conditions changes are negligible and do not affect

Table 5. Accuracy assessment for calibration 1.

Levels	Level 1	Level 2	Level 3	Level 4	Level 5	Level 6
Reference value ($\mu\text{g}\cdot\text{L}^{-1}$)	0.400	1.000	2.000	4.000	6.000	8.000
Maximum acceptable deviation (%)	60%	40%	30%	30%	20%	20%
Precision						
Repeatability standard deviation (S_r)	0.007	0.018	0.028	0.037	0.066	0.069
Intermediate precision standard deviation (S_R)	0.013	0.019	0.027	0.048	0.084	0.113
Repeatability coefficient of variation (CV_r)	1.70%	1.78%	1.39%	0.93%	1.10%	0.86%
Intermediate precision coefficient of variation (CV_R)	3.31%	1.93%	1.35%	1.20%	1.39%	1.41%
Relative uncertainty of precision ($k = 2$)	6.6%	3.9%	2.7%	2.4%	2.8%	2.8%
Trueness						
Estimated average value	0.398	1.002	2.006	3.995	6.026	8.013
Relative bias (%)	-0.56%	0.23%	0.28%	-0.12%	0.44%	0.16%
Maximum acceptable bias (%)	60.0%	40.0%	30.0%	30.0%	20.0%	20.0%
Normalized deviation (ND)	0.201	0.114	0.050	0.031	0.148	0.069
Conclusion: bias is	negligible	negligible	negligible	negligible	negligible	negligible
Accuracy study						
Upper acceptance limit	0.6400	1.4000	2.6000	5.2000	7.2000	9.6000
Upper tolerance limit	0.4241	1.0410	2.0596	4.0912	6.1937	8.2393
Lower tolerance limit	0.3714	0.9636	1.9515	3.8996	5.8590	7.7870
Lower acceptance limit	0.1600	0.6000	1.4000	2.8000	4.8000	6.4000
Conclusion: accuracy is	verified	verified	verified	verified	verified	verified
Fisher test						
Fobserved	0.0004	0.0020	0.0048	0.016	0.0350	0.061
β risk						
β risk (%)	1.37E-08%	1.65E-09%	2.12E-09%	3.70E-10%	5.32E-07%	5.25E-07%
Accuracy profile compared to bias						
Acceptance limit (%) \pm	60%	40%	30%	30%	20%	20%
Bias (%)	-0.56%	0.23%	0.28%	-0.12%	0.44%	0.16%
Upper limit of bias tolerance interval (%)	6.0%	4.1%	3.0%	2.3%	3.2%	3.0%
Lower limit of bias tolerance interval (%)	-7.1%	-3.6%	-2.4%	-2.5%	-2.4%	-2.7%
Estimation of the expanded relative uncertainty						
$U_{\%,k=2} = 2 * \sqrt{(CV_{bias})^2 + (CV_R)^2}$	7%	4%	3%	2%	3%	3%

Table 6. Accuracy assessment for calibration 2.

Levels	Level 1	Level 2	Level 3	Level 4	Level 5	Level 6
Reference value ($\mu\text{g}\cdot\text{L}^{-1}$)	0.40	1.00	2.00	4.00	6.00	8.00
Maximum acceptable deviation (%)	60%	40%	30%	30%	20%	20%
Precision						
Repeatability standard deviation (S_r)	0.007	0.029	0.028	0.034	0.033	0.037
Intermediate precision standard deviation (S_R)	0.017	0.055	0.058	0.109	0.134	0.188
Repeatability coefficient of variation (CV _r)	1.78%	2.84%	1.39%	0.86%	0.55%	0.46%
Intermediate precision coefficient of variation (CV _R)	4.28%	5.46%	2.91%	2.70%	2.22%	2.36%
Relative uncertainty of precision (k = 2)	8.6%	10.9%	5.8%	5.4%	4.4%	4.7%
Trueness						
Estimated average value	0.395	1.006	1.979	4.017	6.023	7.992
Relative bias (%)	-1.37%	0.58%	-1.05%	0.42%	0.38%	-0.10%
Maximum acceptable bias (%)	60.0%	40.0%	30.0%	30.0%	20.0%	20.0%
Normalized deviation (ND)	0.352	0.245	0.726	0.250	0.250	0.048
Conclusion: bias is	negligible	negligible	negligible	negligible	negligible	negligible
Accuracy study						
Upper acceptance limit	0.6400	1.4000	2.6000	5.2000	7.2000	9.6000
Upper tolerance limit	0.4283	1.1155	2.0943	4.2338	6.2906	8.3691
Lower tolerance limit	0.3608	0.8960	1.8636	3.7996	5.7550	7.6152
Lower acceptance limit	0.1600	0.6000	1.4000	2.8000	4.8000	6.4000
Conclusion: accuracy is	verified	verified	verified	verified	verified	verified
Fisher test						
Fobserved	0.0004	0.0108	0.0079	0.0069	0.0052	0.0043
β risk						
β risk (%)	9.20E-07%	9.01E-03%	1.09E-04%	2.40E-05%	5.51E-04%	1.00E-03%
Accuracy profile compared to bias						
Acceptance limit (%)±	60%	40%	30%	30%	20%	20%
Bias (%)	-1.37%	0.58%	-1.05%	0.42%	0.38%	-0.10%
Upper limit of bias tolerance interval (%)	7.1%	11.5%	4.7%	5.8%	4.8%	4.6%
Lower limit of bias tolerance interval (%)	-9.8%	-10.4%	-6.8%	-5.0%	-4.1%	-4.8%
Estimation of the expanded relative uncertainty						
$U_{\%,k=2} = 2 * \sqrt{(CV_{bias})^2 + (CV_R)^2}$	9%	11%	6%	5%	5%	5%

the results. Finally, for each calibration point, the expanded relative uncertainty was evaluated. For calibration 1, the results fluctuate between 7% and 2% going to the lowest to the higher iodine concentration whereas for calibration 2, the uncertainties are larger with values ranging from 11% to 5%. These expanded relative uncertainties are quite satisfactory.

3.3.3. LOD and LOQ Assessment

LOD and LOQ experiments were conducted on ten reagent blank samples (containing sodium thiosulfate, 2% NH_4OH and ^{125}Te). Once analyzed, through the calibration function, iodine concentrations were determined as well as the stan-

dard deviation on all measurements. Then, LOD and LOQ in urine were evaluated taking into account the dilution factor used (63-fold) for the samples. LOD was estimated to $0.39 \mu\text{g}\cdot\text{L}^{-1}$ for calibration 1 and $0.35 \mu\text{g}\cdot\text{L}^{-1}$ for calibration 2.

About LOQ, the values found were $1.30 \mu\text{g}\cdot\text{L}^{-1}$ and $1.18 \mu\text{g}\cdot\text{L}^{-1}$ for calibration 1 and 2, respectively.

Literature reported on iodine LOD in urine by ICP-MS are quite various. Among them, Allain *et al.* determined a LOD (calculated as twice the standard deviation of the background signal) in urine of $1.6 \mu\text{g}\cdot\text{L}^{-1}$ with a 10-fold dilution in acid media using external calibration method [19]. Other authors have carried out measurements on twenty urine samples with a 50-fold dilution factor in alkaline media using external calibration and matrix-matched calibration solutions [28]. The LOD estimated was $1 \mu\text{g}\cdot\text{L}^{-1}$. Finally, according to another recent publication, a LOD was determined at $4 \mu\text{g}\cdot\text{L}^{-1}$ on blank samples in acid media [29]. Compared to these works, our two proposed methods exhibited very satisfactory LOD values.

To complete the validation process, the verification of the LOQ previously measured ($0.02 \mu\text{g}\cdot\text{L}^{-1}$ considering the sample dilution factor) was performed. For calibration 1, as the previously mentioned value could not be verified, we chose the value $0.2 \mu\text{g}\cdot\text{L}^{-1}$ while remaining below the lower calibration standard solution ($0.4 \mu\text{g}\cdot\text{L}^{-1}$).

The results for the two methods are presented in **Table 7**. For each calibration methods, the two inequalities were verified.

3.3.4. Selectivity

Before analyzing the results, as for accuracy, the Cochran C test and the Grubbs G test were employed at each addition and for both methods. All data followed a Gaussian distribution. Therefore, selectivity analysis could be performed. The results are reported in **Table 8**.

For calibration 1, the objective was to reach the values 0.67, 1.33 and $2.43 \mu\text{g}\cdot\text{L}^{-1}$. The achieved recovery rates were respectively 98.3%, 95.4% and 93.1%. The results are considered quite sufficient since they are within 90% - 110% of expected value.

Table 7. Results of the verification of LOQ calculated before for calibration 1 and 2.

	Calibration 1	Calibration 2
LOQ value	$0.2 \mu\text{g}\cdot\text{L}^{-1}$	$0.02 \mu\text{g}\cdot\text{L}^{-1}$
\bar{Z}_{LOQ}	0.2097	0.0220
S_{LOQ}	0.030	0.004
$\bar{Z}_{\text{LOQ}} - 2 S_{\text{LOQ}}$	0.149	0.0141
LOQ - 60% LOQ	0.080	0.008
$\bar{Z}_{\text{LOQ}} + 2 S_{\text{LOQ}}$	0.270	0.030
LOQ + 60% LOQ	0.320	0.032
Conclusion	verified	verified

Table 8. Specificity results for calibration 1 and 2.

Calibration method	N° experiment	Spike concentration expected ($\mu\text{g}\cdot\text{L}^{-1}$)	Spike concentration observed ($\mu\text{g}\cdot\text{L}^{-1}$)			Yield (%)			Yields mean (%)	Recovery rate (%)
			1	2	3	1	2	3		
	1	0.67	0.682	0.646	0.674	101.79	96.37	100.61	99.59	98.3
	2		0.689	0.685	0.682	102.78	102.21	101.82	102.27	
	3		0.660	0.660	0.665	98.45	98.47	99.25	98.72	
	4		0.632	0.678	0.634	94.30	101.22	94.69	96.74	
	5		0.669	0.669	0.623	99.91	99.81	92.95	97.56	
	6		0.689	0.641	0.631	102.81	95.60	94.19	97.53	
	7		0.668	0.622	0.637	99.63	92.80	95.10	95.84	
Addition standard	1	1.33	1.241	1.231	1.209	95.48	94.67	93.01	94.39	95.4
	2		1.226	1.275	1.230	94.27	98.08	94.60	95.65	
	3		1.247	1.286	1.298	95.88	98.95	99.82	98.22	
	4		1.219	1.153	1.221	93.78	88.68	93.95	92.13	
	5		1.202	1.221	1.203	92.47	93.91	92.53	92.97	
	6		1.254	1.275	1.272	96.49	98.07	97.88	97.48	
	7		1.246	1.273	1.271	95.81	97.94	97.75	97.17	
	1	2.43	2.205	2.229	2.207	90.73	91.72	90.82	91.09	93.1
	2		2.199	2.209	2.210	90.50	90.93	90.95	90.79	
	3		2.039	2.041	2.095	83.92	83.99	86.21	84.71	
	4		2.327	2.312	2.365	95.78	95.14	97.32	96.08	
	5		2.224	2.393	2.240	91.54	98.48	92.18	94.07	
	6		2.337	2.424	2.372	96.17	99.76	97.59	97.84	
	7		2.340	2.322	2.418	96.29	95.57	99.52	97.13	
External calibration	1	1.6	1.817	1.857	1.876	113.56	116.04	117.26	115.62	107.1
	2		1.553	1.551	1.563	97.06	96.91	97.69	97.22	
	3		1.611	1.582	1.573	100.71	98.86	98.33	99.30	
	4		1.542	1.547	1.539	96.37	96.69	96.17	96.41	
	5		2.105	2.035	1.942	131.59	127.20	121.36	126.71	
	1	6.4	7.170	7.310	7.296	112.03	114.22	114.01	113.42	108.4
	2		6.723	6.767	6.675	105.05	105.74	104.30	105.03	
	3		6.270	6.283	6.268	97.97	98.16	97.94	98.02	
	4		6.485	6.478	7.247	101.33	101.22	113.24	105.26	
	5		7.825	7.444	7.856	122.26	116.31	122.75	120.44	

For calibration 2, the values $1.6 \mu\text{g}\cdot\text{L}^{-1}$ and $6.4 \mu\text{g}\cdot\text{L}^{-1}$ were investigated and the estimated average yields were 107.1% and 108.4%. These results are more and less 10% from the value targeted which is acceptable.

3.3.5. Cross-Contamination Study

Possible contamination may occur and affect the samples during the analysis. An evaluation of the inter-sample contamination is therefore required to ensure the reliability of the results. The results were 0.03% and 0.15% for calibration 1 and 2, respectively. Thus, as the performance criterion is set at 5%, the results are more than suitable.

3.3.6. Urinary Iodine Estimation in Rats

The concentration of urinary iodine of twelve rat urine samples (six control rats and six treated rats) was determined by ICP-MS using both calibration methods previously validated.

The measured values mean results are shown in **Table 9**.

U_1 refers to overall uncertainty for calibration 1 (u_1 , u_2 and u_3 correspond to uncertainties for the three replicates) and U_2 refers to overall uncertainty for calibration 2 (u_1 , u_2 and u_3 correspond to uncertainties for the three replicates).

A high discrepancy is observed in iodine concentration in urines from treated rats. For rats n° 100, 120 and 140, 24-hour urine samples were collected 24 hours after gavage which explains the high concentrations of iodine. Indeed, after absorption, iodine is rapidly excreted [30] whereas, for the rats n° 102, 122 and 142, 24-hour urine samples were collected 48 hours after gavage which justifies significantly lower iodine concentrations.

To compare the results of both calibration methods, the non-parametric Wilcoxon W test and the Z-score calculation were used. For each sample, as noticed in **Table 9**, Wilcoxon W test concluded to no significantly difference between the results. Consequently, according to this test, both calibration methods are thus comparable.

Relating to the other way to investigate the results, namely Z-score, the interpretation of this parameter is as follows. When the calculated Z-score is between -2 and 2, the result is considered as satisfactory whereas if a Z-score value is found between -3 and -2 or 2 and 3, the result is seen as doubtful. Considering the obtained values, the results are quite acceptable except for the treated rat n° 122 (with a Z-score of -2.23).

Table 9. Results of urinary iodine in rats by both calibration methods.

Rat	Measured values mean ($\mu\text{g}\cdot\text{L}^{-1}$) \pm uncertainty calibration 1		Measured values mean ($\mu\text{g}\cdot\text{L}^{-1}$) \pm uncertainty calibration 2		Wilcoxon test ^b	Z-score ^c
Control - n°99	973.45	\pm 56.05	967.14	\pm 60.52	ns	0.08
Control - n°101	1118.75	\pm 76.74	1197.92	\pm 57.69	ns	-0.82
Control - n°119	813.83	\pm 69.35	955.04	\pm 45.80	ns	-1.70
Control - n°121	692.87	\pm 59.92	832.73	\pm 51.81	ns	-1.77
Control - n°139	688.42	\pm 45.07	672.31	\pm 36.09	ns	0.28
Control - n°141	676.21	\pm 49.87	697.63	\pm 38.15	ns	-0.34
Treated - n°100	15 293.30	\pm 792.79	15 706.46	\pm 621.33	ns	-0.41
Treated - n°102	1108.49	\pm 65.47	1170.79	\pm 42.48	ns	-0.80
Treated - n°120	13 534.95	\pm 1027.94	12 502.77	\pm 473.37	ns	0.91
Treated - n°122	1223.46	\pm 76.47	1428.09	\pm 50.66	ns	-2.23
Treated - n°140	14 396.92	\pm 1539.12	13 393.47	\pm 490.05	ns	0.62
Treated - n°142	1064.42 ^a	\pm 87.25	1082.85	\pm 40.43	ns	-0.19

^aDue to technical problem, mean is measured only on two replicates; ^bns means no difference statistically

significant; ^cZ-score formula = $\frac{m_1 - m_2}{\sqrt{U_1^2 + U_2^2}}$ with $U_1 = \sqrt{\frac{u_1^2 + u_2^2 + u_3^2}{3}}$ and $U_2 = \sqrt{\frac{u_1^2 + u_2^2 + u_3^2}{3}}$.

These two tests allow us to conclude that both calibration methods are comparable.

These analyses in control rats were the opportunity to estimate the urinary iodine in this animal species because as far as we know, the information on this biological parameter is of great rarity. According to our findings, urinary iodine concentration for 3-month-old male Wistar rat is comprised between 672.31 $\mu\text{g}\cdot\text{L}^{-1}$ and 1197.92 $\mu\text{g}\cdot\text{L}^{-1}$ and highlights the great intra-rat variability. Even though this information was widely available in the literature, there would be too much uncertainty related to the diet, the gender or the species to compare the data.

4. Conclusion

Taking into account the results of each criterion, we can conclude that both calibration methods for urinary iodine determination by ICP-MS are validated as analyzed performances fully complying with all the requirements we had previously imposed, with maybe greater satisfaction for calibration 1 (especially considering its very good linearity, excellent accuracy with quite low expanded standard uncertainties). Given the validation results, both calibration methods can be used. Nevertheless, if focus is given on practical or logistical issues, external calibration method should be preferred because of an overall analysis time significantly shortened and therefore a quicker delivery of the results. To these advantages, we can also add the lowest cost for lab materials. Otherwise, the recent acquisition of an ICP-MS “iCAP-Q” (Thermo Fisher ScientificTM, France) enabled us to considerably optimize the analysis time (factor 9 between ICP-MS “X series II” and “iCAP-Q” in external calibration). In fact, the device is equipped with a FAST system which starts rinsing even though the sample is being introduced into the ICP-MS. Finally, when applied to iodine measurement in rat urine samples, both calibration methods are in good agreement. Thus, we can conclude that both procedures have been validated successfully ensuring the reliabilities of future results for rodent’s experiments.

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