Standard operating procedure

for assessment of mercury in urine
(*sampling, analysis of total mercury, interpretation of results*)

2018

Abstract

This standard operating procedure describes the process of assessing exposure to mercury through human biomonitoring using urine as a biological matrix. Sampling of urine, analysis of total mercury and interpretation of results are detailed in this document.

Key words

Mercury - analysis
Mercury - urine
Methylmercury Compounds - analysis
Urine - chemistry
Biomarkers - analysis
Maternal Exposure
Maternal-Fetal Exchange
Infant, Newborn
Environmental Exposure

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Abbreviations

CVAAS   cold vapour atomic absorption spectrometry

HBM   human biomonitoring

Hg   mercury

ID   identity

LOD   limit of detection

LOQ   limit of quantification

SG   specific gravity

SOP   standard operating procedure

Introduction: urine as a matrix for mercury human biomonitoring

Mercury (Hg) is a naturally occurring element that is distributed throughout the environment by both natural and anthropogenic processes. It is persistent in the environment and is found in various chemical forms, namely elemental mercury, inorganic mercury (Hg^{2+} compounds) and organic mercury (mainly methylmercury, MeHg) *(1)*.

The primary targets for mercury toxicity are the nervous system, kidneys and cardiovascular system. Nervous system effects are the most sensitive toxicological endpoint observed following exposure to elemental mercury and methylmercury, whereas kidney damage is the key endpoint in exposure to inorganic mercury compounds *(1,2)*.

Exposure to elemental or inorganic mercury occurs due to mercury spills, dental amalgams, inhalation indoors due to broken thermometers and mercury-containing bulbs, the use of some skin-lightening creams and soaps, the presence of mercury in some traditional medicines, the use of mercury in cultural practices and occupational exposure *(3–5)*.

Exposure can be estimated by measuring pollutant levels in various human matrices, such as hair, blood or urine, all of which are useful tools for assessing mercury exposure in individuals and populations *(3,6–8)*.

Urinary mercury levels are usually considered to be the best measure of recent exposure to inorganic mercury or elemental mercury vapour, as urinary mercury is thought to most closely indicate the mercury levels present in the kidneys *(3)*.

Urine is easy to collect and is available in larger amounts than other biological matrices. Spot urine samples are usually employed instead of 24-hour samples as the latter are more uncomfortable to collect and are more likely to become contaminated due to continuous opening of the vessel.

The disadvantage of spot urine samples is the variability in the volume of urine produced and the fact that the concentration of endogenous and exogenous chemicals can vary significantly from void to void depending on the hydration status, time of last urination, etc. Consequently, spot samples need a dilution adjustment. Several methods, such as creatinine adjustment or specific gravity, can be used to adjust the urinary biomarker concentration *(9–11)*.

Although spot urine samples can be collected at any time of day, a first morning urine sample is recommended as otherwise the target biomarker may be below the limit of quantification (LOQ) due to sample dilution. A further possibility is to collect samples after at least five hours without urination.

1. Sampling of urine

1.1. Scope of the method

The method of collecting urine samples described in this standard operating procedure (SOP) allows analysis of mercury concentrations, and covers all pre-analytical phases of mercury human biomonitoring (HBM) using urine. Following the sampling procedure detailed in this SOP enables field technicians to properly collect and handle the biological samples before they are analysed in the laboratory.

1.2. Safety precautions

Urine samples will be collected by the recruited women themselves. However, when working with urine (aliquoting or making other manipulations) universal precautions for working with biological materials should be followed.

- Wear gloves, a laboratory coat and safety glasses while handling human bodily fluids or tissue.
- Place disposable plastic, glass and paper (e.g. pipette tips, autosampler tubes and gloves) that come in contact with human biological fluids, such as urine, in a biohazard autoclave bag.
- Keep these bags in appropriate containers until they are sealed and autoclaved.

1.3. Materials required

Table 1 shows the materials required for sampling and for pre-treatment of the sampling material.

Table 1. Materials for urine sampling

Material	Rationale	Alternative
Extra pure 65% nitric acid	Used to clean the vessels in order to eliminate background metal contamination.	
Purified water	Used in the cleaning process.	Bidistilled water
Containers	3 different tanks for the cleaning process; 1 for the acid solution and the other 2 for water.	
Urine vessels (see below)	Vessels that can be closed reliably. The volume of the vessel depends on the amount of urine required for analysis and biobanking (if planned).	
Acid-resistant gloves	A safety measure.	
Labels	Samples must be unequivocally identified.	Writing the ID code directly on the vessel with a permanent marker pen
Permanent marker pen	Not essential but very useful to mark the minimum amount of sample that should be collected.	Any other writing material which ensures that the mark remains clearly legible
Filter paper	Used during vessel washing.	
Zip-lock plastic bags	Used to further isolate the vessel.	Any other type of bag
Isothermal packaging	Urine samples have to be kept at 4 °C until arrival at the laboratory.	
Freeze-safe labels for sample	Used for labelling of samples.	
2M sulfamic acid	For the purpose of preventing loss of mercury from the urine before analysis.	

ID = identity.

For mercury analysis, 2M sulfamic acid should be added prior to urine sampling in the proportion of 10 µL of preservative solution per 1 mL of urine (e.g. for a tube containing 50 µL of preservative, up to 5 mL of urine can be added for urine mercury analysis).

1.4. Preparation/pre-treatment of the sampling material

The vessels employed for urine sampling must be pre-cleaned to eliminate the background metal contamination. All vessels and their lids should be washed with nitric acid solution according to the following procedure. Note that pre-washing of the vessels should be performed in a chemical fume hood according to good laboratory practice, following the laboratory's safety guidelines and whilst wearing protective equipment.

1. Mark the different containers according to the solution contained in them: 10% nitric acid; rinse tank 1; rinse tank 2.
2. Place them in the chemical fume hood.
3. Prepare the dilute acid solution from extra pure 65% nitric acid and purified water. (Note: 18 L of acid solution (2.8 L 65% nitric acid and 15.2 L of bidistilled water) is required to clean approximately 240 100 mL vessels. The acid solution can be used for up to one month after preparation).
4. Fill the tanks with the corresponding solution.
5. Open the vessels and place them in the tank containing the acid solution together with their lids (overnight or for at least for three hours). Ensure that the vessels and lids are completely immersed (Photo 1).
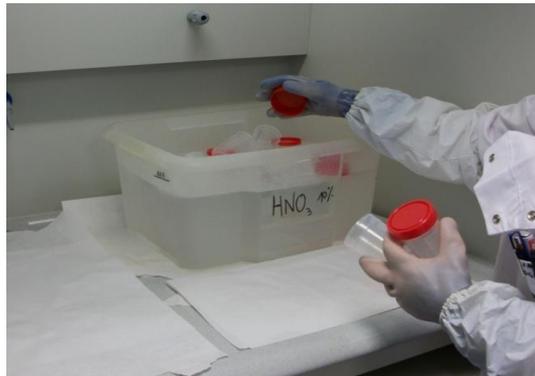
Photo 1. Placing the vessels and lids in the acid
solution
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6. Remove the vessels from the acid tank and place them in the first tank of purified water, shaking for 2–3 minutes. Then move the vessels and lids to the second tank, again shaking for 2–3 minutes (Photo 2).
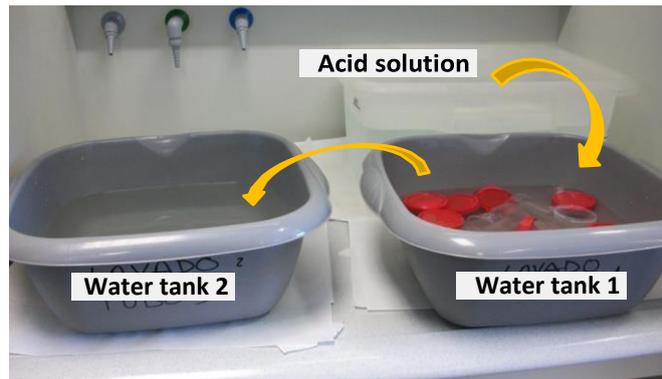
Photo 2. Rinsing the vessels in purified water tanks
© Instituto de Salud Carlos III

7. Remove the vessels and lids from the second water tank and place them face down on a clean sheet of filter paper inside the chemical fume hood to dry (Photo 3).

Photo 3. Drying the vessels and lids in the fume hood
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8. Replace the lids on the vessels, then mark the vessels to indicate the minimum volume required (Note: this step is optional but is very useful to avoid the collection of samples with insufficient volume). Place each washed vessel into a zip-lock plastic bag (photos 4a and b).

Photos 4a and b. Marking the minimum volume (a) and placing the vessels in a zip-lock bag
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The presence of contamination after the washing procedure should be checked by selecting 5% of the washed vessels at random and filling them with purified water. After shaking for 10 minutes, one aliquot should be taken from each vessel to determine the mercury concentration.

Finally, if the vessels come from different batches, information regarding the batch of vessels sent to each sampling centre should be recorded.

1.5. Sampling procedure

Ideally, urine vessels should be provided to the volunteers by the fieldworkers in advance in order to allow them to collect the first morning urine. (Alternatively, urine samples can be collected from the mother at admission to the maternity ward, prior to child birth.) Each vessel should be accompanied by detailed written instructions on how to collect the urine sample (see an example in Annex 1). In addition to these written instructions, fieldworkers should explain personally to the volunteers how to collect the sample and clarify any questions and doubts. Questionnaires for urine samples (Annex 2) should be administered at the time of sampling.

Urine samples collected should be kept at 4 °C until their arrival at the laboratory. Alternatively, urine samples can be aliquoted in the maternity unit and frozen at -20 ℃. In this case, samples have to be kept frozen during transportation to the laboratory.

Note. Control blank samples should be used regularly (at least one blank sample in each maternity unit). Containers for blank samples should be opened in the maternity ward and manipulated exactly like containers for regular samples but without collecting the sample. This allows assessment of potential sample contamination at the sampling location.

1.6. Labelling

Containers for urine samples can be labelled in two different ways.

- In advance, after the washing procedure: a label with the identity (ID) code and a space to note the sampling date is affixed.
- After sample collection: immediately after the volunteer delivers the sample to the fieldworker, the container should be labelled with the ID code and sampling date.

1.7. Transportation and conservation of the sample

Urine samples must be kept at 4 °C until their arrival at the laboratory, where they will be aliquoted and analysed or stored until analysis. (Alternatively, the samples can be aliquoted and frozen in the maternity unit.) Furthermore, urine samples must be transported in compliance with the relevant shipping regulations for biological material. Photo 5 illustrates appropriate isothermal packaging for sample transport.
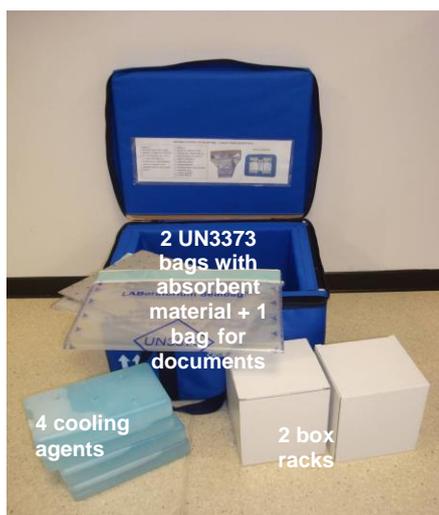
Photo 5. Example of isothermal packaging
© Instituto de Salud Carlos III

1.8. Sample reception

The following points should be checked upon receipt of urine samples.

- Condition of sample transportation and storage (samples transported and stored at high temperature cannot be accepted).
- Use of preservatives during sampling (containers with 2M sulfamic acid should be used for urine sampling for mercury analysis).
- The package must be correctly sealed and must not have been manipulated (Note: a security seal can be placed on the package at the sampling site).
- All samples listed in the registry of collected samples should be contained in the package.
- All samples must be accompanied by the corresponding documents (questionnaires, etc.).
- All samples and documents received must be properly identified with the corresponding ID code.
- The samples must have been collected properly (sufficient volume).
- The transportation container should not be contaminated.

Annex 3 contains a sample reception sheet for urine samples. The items in this document may vary according to the requirements for sample conservation or analyte stability/conservation.

Note. If field blanks (for example, vessels with purified water) have been employed, they should be checked and registered in the same manner as the other samples.

1.8.1 Sample acceptance/rejection criteria

The criteria for accepting or rejecting a sample should be defined in advance and applied during sample reception. These criteria should focus on transport conditions, attached

documentation, integrity of the packaging, correct identification, amount of sample (sufficient for analysis and biobanking if samples will be stored for other research purposes). In order to follow a unique procedure and apply the same criteria to all samples received, the plan illustrated in Fig. 1 can be applied.
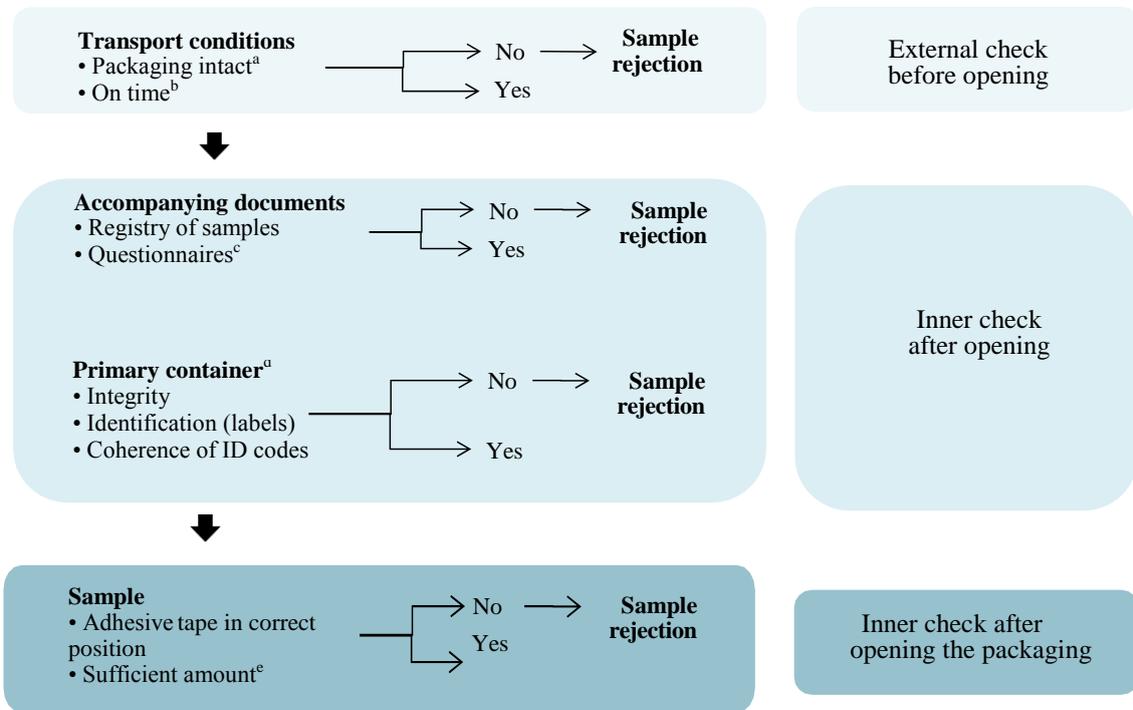
Fig. 1. Plan for receipt of samples

[a] The package must be correctly sealed and must not have been manipulated.

[b] The maximum time between sample collection and its arrival at the laboratory should be defined beforehand (e.g. five days after sampling). This will depend on the time that samples can be kept in the required conditions or limitations due to the target biomarker.

[c] If one or more of the questions in the questionnaires are crucial for results interpretation or are an inclusion/exclusion criterion, this should be verified.

[d] The conditions of the vessels should be checked. All samples must be properly identified and the consistency between sample ID codes and questionnaires should be checked.

[e] The amount of sample is a critical point. If the amount of sample is insufficient to perform the chemical analysis, the sample should be rejected.

1.9. Sample aliquoting/preparation

Sample aliquoting can be conducted in the hospital or in the laboratory after transportation from the hospital. Urine samples should be aliquoted in accordance with good laboratory practice, following the laboratory's safety guidelines and whilst wearing protective equipment. The number and volume of aliquots required should be estimated in order to avoid freeze-thaw cycles. The laboratory performing the analysis should be consulted to establish the aliquot volume required and the minimum volume required for analysis.

The full list of tubes for aliquoting is as follows.

1. Tube U1 (mercury)
   a. 5 mL urine: pour (no pipetting!) into a pre-treated plastic metal-free urine container with sulfamic acid added prior to sampling. Mix the urine well after adding it to the vial.
   b. Freezing and storage at -20 °C.
2. Tube U2 (creatinine)
   a. 5 mL urine: pour into a 15 mL polypropylene tube.
   b. Freezing and storage at -20°C.
3. Additional tubes: the rest of the urine can be poured into separate tubes for additional analyses. It is recommended to also store polypropylene tube(s) in fractions of 10 mL or 40 mL in the biobank at -80 °C.

Note. Ensure that the aliquots are homogeneous by shaking the original sample between aliquots.

1.10. Storage and conservation

Samples to be stored for more than one month should be frozen. Since urine contains many inorganic salts, even fresh urine may generate precipitate. Thus, the sample must be homogenized by shaking before analysis. A method also exists where the solubility of the salts is increased by lowering the pH of the urine sample by adding a small amount of hydrochloric acid. Take steps to ensure that microorganisms do not proliferate, as they may cause inorganic mercury to reduce to mercury vapour, which will escape and be lost. It is believed that the average mercury level in the urine of the general population in a region without any particular mercury exposure is less than 10 ng/mL. Mercury stability has been demonstrated for one year at -20 °C.

In general, urine specimens are transported and stored at -20 °C. Sample storage procedures should be established in order to control the sample location, number of aliquots remaining, etc. Upon receipt, freeze the specimens at -20 °C until the time for analysis. The analyst must put the remaining samples in the freezer after analytical aliquots are taken and refreeze them at -20 °C. Samples that are thawed and refrozen several times will not be compromised even if preservatives were added for storage.

1.11. Quality control

1.11.1. Traceability

Traceability of the sample throughout the study is crucial, therefore this aspect should be guaranteed. As noted above, correct labelling of the samples and related documents is essential, but it is also necessary to be able to link the sample with the information provided by the volunteer. It is strongly recommended that a log of samples be maintained (Annex 3). To this end, a database should be designed where this information can be stored. Access to this file or document must be restricted whenever it contains confidential personal information.

If more than one ID code is associated with a sample, for example aliquots from the samples can have different codes, or if an internal code has to be assigned when samples arrive at the laboratory, all these codes should be recorded in the database.

2. Analysis of total mercury in urine

The method described in this SOP is suitable for the determination of total mercury in human urine in a general population with low exposure to mercury and for occupationally exposed humans. The method is based on acid digestion, reduction and measurement by cold vapour atomic absorption spectrometry (CVAAS). The method is simple and sensitive. It is designed to be suitable for an instrument that requires simple maintenance and has been promoted by the National Institute for Minamata Disease (Japan) *(12)*.

Where laboratories have other equipment for the detection of mercury in acid digested samples it is advisable to follow the instructions provided by the instrument producers. The instructions for sampling and sample handling provided in this SOP are fit for purpose regardless of the instrumentation used for mercury detection. The limit of detection (LOD) and LOQ should be checked to ensure that they are suitable for human urine samples.

2.1. Scope of the method

The described procedure refers to the treatment and processing of the sample after sub-aliquots are taken for mercury analysis. Concentrations of total mercury in urine of a non-exposed population are normally in the range of 0.1–5 ng/mL. In cases of exposure to inorganic and elemental mercury, values up to 10 ng/mL have been reported; however, at workplaces levels higher than 50 ng/mL frequently occur. The described method can cover all the ranges normally reported in general populations, as well as in occupational exposure settings.

2.2. Technical principle

Urine samples are digested by acids and mercury is detected by CVAAS. This process is based on the reduction of ionic mercury in the solution to its elemental state and its subsequent transfer into the absorption cell of the mercury analyser for measurement at 253.7 nm. The measurement process is based on the open air flow system, which requires clean ambient air as a carrier gas, making the apparatus easy to operate.

Many detectors are available today for the measurement of mercury and are based either on atomic absorption or atomic fluorescence spectrometry. The procedures used by the laboratories must comply with the instructions provided by instrument producers *(13,14)*. See also the *Standard operating procedures for the determination of total mercury in hair, blood and urine by the alternative method*.

2.3. Safety precautions

Follow universal precautions: wear gloves, a laboratory coat and safety glasses while handling human bodily fluid or tissue. Place disposable plastic, glass and paper (e.g. pipette tips, autosampler tubes and gloves) that come into contact with human biological fluids, such as urine, in a biohazard autoclave bag. Keep these bags in appropriate containers until

they are sealed and autoclaved.

When the work is finished, wipe down all work surfaces where human biological fluid was handled using a 10% (v/v) sodium hypochlorite solution or equivalent. The use of the foot pedal on the Micromedic Digiflex is recommended because it reduces analyst contact with work surfaces and also keeps the hands free to hold specimen cups and autosampler tubes. Dispose of all biological samples and diluted specimens in a biohazard autoclave bag at the end of the analysis in accordance with guidelines for disposal of hazardous waste.

2.4. Equipment, materials and solutions

2.4.1. Equipment

The method described in this SOP includes the following: reduction of inorganic mercury ions in the sample test solution with stannous chloride to generate elemental mercury vapour; and the introduction of mercury vapour into the photo-absorption cell of the mercury analyser for the measurement of absorbance at 253.7 nm. This method uses a circulation-open air flow system as shown in Fig. 2. The apparatus constitutes a closed system and comprises a diaphragm pump, reaction vessel, acid gas trap, moisture trap (ice bath) and a 4-way cock.

During its operation, the elemental vapour generated by the addition of stannous chloride is circulated via the 4-way cock at a flow rate of 1–1.5 L/min for 30 seconds to allow the mercury vapour to come to an equilibrium between gaseous and aqueous phases. The 4-way cock is then rotated by 90° to introduce the gas phase into the photo-absorption cell all at once. The measurement is completed within one minute per sample with this apparatus, which can measure even 0.1 ng of mercury with high accuracy and precision.

2.4.2. Materials

The following materials are required for analysing total mercury in urine:

- mercury analyser: Model Hg-201 Semi-Automated Mercury Analyser;
- hot plate: capable of attaining a surface temperature of 250 °C;
- sample digestion flask: 50 mL thick-walled volumetric flask made of Pyrex (150 mm total height, 13 mm inlet diameter);
- volumetric flasks: 10, 100 and 1000 mL;
- measuring pipettes: 0.2, 0.5, 1.5 and 10 mL; automatic pipettes can also be used (range 0.1–10 mL);
- centrifuge;
- multi-flow meter: V4-type flow meter multi-kit.

Fig. 2. Schematic diagram of reduction/cold vapour atomic absorption spectrometry (circulation-open air flow system)
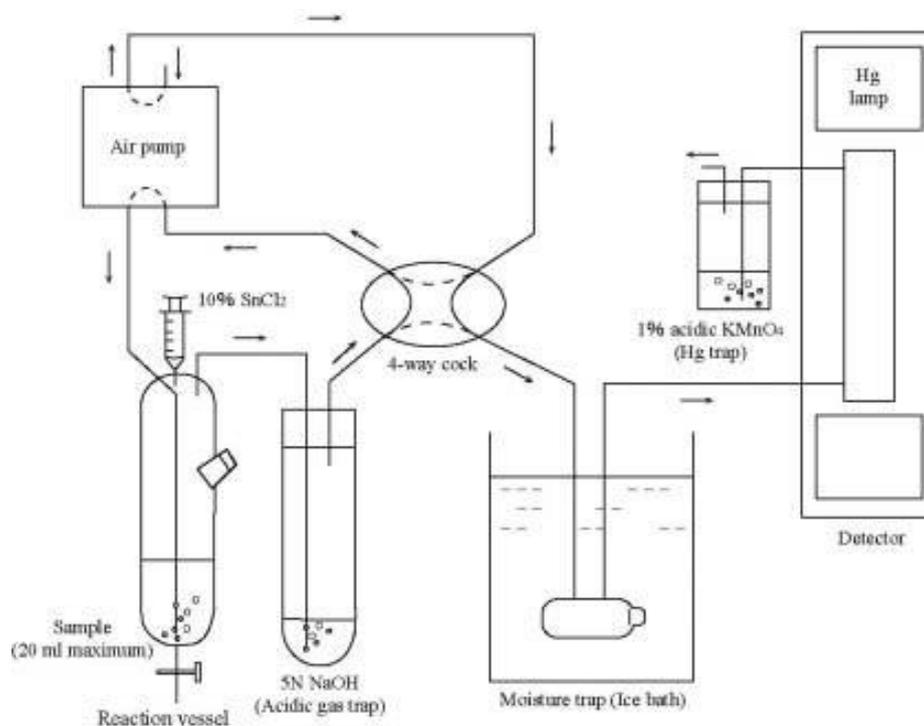
Source: Akagi 1997 *(12)*

2.4.3. Reagents and chemicals

The following reagents and chemicals are required for analysing total mercury in urine.

- Nitric acid-perchloric acid (1+1): mix 100 mL of perchloric acid (for measurement of toxic metals) into 100 mL of nitric acid (for measurement of toxic metals). Store in a cool dark place.
- Sulfuric acid (for measurement of toxic metals).
- Distilled water: distil deionized water and store in a clean glass container.
- Hydrochloric acid (analytical grade).
- 2M sulfamic acid: partially fill a pre-screened or pre-acid-washed 50 mL polypropylene centrifuge tube with bidistilled water. Add 10 g of sulfamic acid. Fill to the 50 mL mark with bidistilled water. Dissolve the sulfamic acid by mixing well (use of a vortexer, or warm water bath is helpful in this process). Store at room temperature. Expiration is one year from preparation.
- 10% tin (II) chloride solution: dissolve 10 g of tin (II) chloride dihydrate (analytical grade), in 9 mL of hydrochloric acid and dilute to 100 mL with distilled water. Aerate with nitrogen gas (100 mL/min, 20–30 minutes) to expel any mercury from the solution.

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- 5M sodium hydroxide: dissolve 20 g of sodium hydroxide (analytical grade) in distilled water to make a final volume of 100 mL.
- 0.1M sodium hydroxide: dilute 5N sodium hydroxide 50-fold with distilled water.
- 2M sulfuric acid: gradually add 30 mL of sulfuric acid (for measurement of toxic metals) to distilled water to make a final volume of 1000 mL.
- 0.5% potassium permanganate solution: dissolve 0.5 g of potassium permanganate (analytical grade) in distilled water to make a final volume of 100 mL. This is used for cleaning the glass ware.

2.4.4. Calibration standards

Inorganic mercury standard solution

Weigh out 13.5 mg of mercury (II) chloride (standard) in a 100 mL volumetric flask, dissolve in 4 mL of nitric acid-perchloric acid (1+1) and 10 mL of sulfuric acid added in turn, and top up to the mark with distilled water to make a stock mercury solution (1 mL of the stock mercury solution = 100 µg mercury). The stock mercury solution obtained in such way will be stable for several years if sealed and stored in a cool dark place. At every use, the stock solution is diluted 10 000 times with the above blank test solution to make a mercury standard solution (1 mL of this solution = 0.010 µg mercury). This should be done in two consecutive steps. Dilutions should be made at an ambient temperature of 20–23 ℃.

Mercury standard solution

A mercury standard solution (SRM 3177) prepared from high-purity mercury (II) chloride is available from the National Institute of Standards and Technology (NIST), for calibration purposes. A unit of this material consists of five borosilicate glass ampoules, with each ampoule containing approximately 10 mL of solution. A certified value is assigned for mercury, with a nominal mass fraction of 1 mg/g. Working standard solutions are prepared by appropriate dilutions by a factor of 10 000. The working calibration solution is prepared in two steps, to obtain a concentration of 0.010 µg/mL.

2.5. Calibration

The multi-point calibration curve method is not always required because the calibration curve is linear across a wide concentration range. Therefore, a three-point calibration curve method is used. In addition to the blank solution, the most suitable concentration of the standard test solution should be chosen (e.g. 0.01, 0.03 or 0.05 µg mercury/50 mL) for a total mercury measurement with a peak height close to that of the sample test solution. In this case, the same volume of both the standard test solution and sample test solution should be used during the measurements. This will facilitate quantification.

2.6. Procedure

2.6.1. Acid digestion

Fig. 3 illustrates the procedure for determination of total mercury in urine. Put 2 mL of

nitric acid-perchloric acid (1+1) and 5 mL of sulfuric acid into a sample digestion flask beforehand. Gradually add a known volume (usually 2 mL) of the urine sample while stirring slowly. Follow the same steps to make a blank test solution and a standard test solution.

Each sample should be prepared in duplicates. Blank solution is prepared in the same way as the sample, except no sample is added to the blank flasks. Standard test solutions are also prepared in the same way as the sample, except that instead of urine samples, standard solution is added to the flasks. At least three calibration points are needed, normally covering the range of 0.5–5 ng/mL.

The sample flasks should be heated for 30 minutes on a hot plate at 200–230 ºC. Once the flasks have cooled, add distilled water to make a fixed volume of 50 mL, mix well and use the resulting solutions as the sample test solutions.

Fig. 3. Determination of total mercury in urine

Sample digestion flask

HNO_3-$HClO_4$ (1+1), 2 mL
H_2SO_4, 5 mL

Urine samples, 2 mL

Add dropwise while swirling slowly
Heat at 200–230 ^{0}C for 30 min

Digested samples

Cool
Top up to 50 mL with distilled water

Test solution, a fixed volume (usually 5 L

10% $SnCl_2$ solution, 1 mL
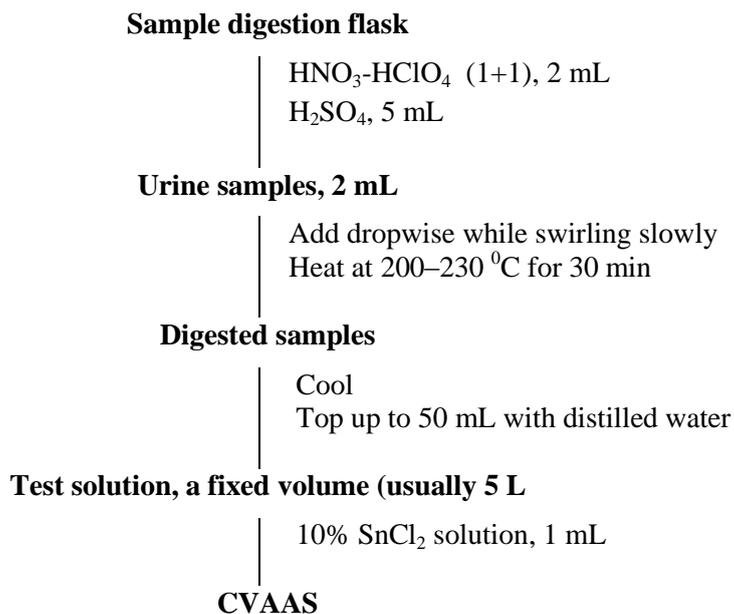
CVAAS

CVAAS = cold vapour atomic absorption spectrometry; H_2SO_4 = sulfuric acid; HNO_3-$HClO_4$ = nitric acid-perchloric acid; $SnCl_2$ = tin (II) chloride.

2.6.2. Measurement

The automated apparatus used for this process is commercially available as a Model Hg-201 Semi-automated Mercury Analyser.

Gently transfer known volumes of test solution (usually 5 mL, up to a maximum of 10 mL) to the reaction vessel of the mercury analyser, add mercury-free water up to the 20 mL mark and

apply the stopper. Add 1 mL of 10% tin (II) chloride in 1N hydrochloric acid solution with the accessory dispenser and push the start button. The diaphragm pump will run and the generated elemental mercury vapour will be circulated through the 4-way cock between the reaction vessel and the acidic gas trap for 30 seconds until equilibrium between gaseous and aqueous phases is reached. Acidic gas generated from the sample test solution is collected in the alkaline solution. After 30 seconds, the 4-way cock will turn automatically by 90°, allowing the introduction of mercury vapour into the photo-absorption cell through an ice bath for measurement of the absorbance. The readings of the recorder will increase sharply and decrease with a sharp peak. When the recorder reading begins to decrease, open the cock on the lower part of the reaction vessel to discard the solution inside, close it again, and allow it to aerate until it returns to the baseline. Push the reset button to start the next measurement. Each of the blank test solutions should be measured first. After that standard test solutions should follow. If the calibration curve is acceptable, sample test solutions can be measured.

Note. The equilibrium concentration between the aqueous phase and the gas phase of reduced and vaporized mercury vapour may differ depending on the acid concentration and volume of the sample test solution at measurement. Therefore, the blank test solution is used for dilution of the sample test solution, and both the sample test solution and the standard test solution are measured under the same conditions in every respect (acid concentration and volume).

2.7. Calculation of the analytical results

The peak heights (mm) obtained after measurement of known volumes of the blank, the standard and the sample test solutions (or their diluted solutions) are labelled P_{blank} (blank), P_{std} (standard) and P_{sample} (sample), respectively. The total mercury concentration in the sample is calculated according to the following formula.

$$c_{sample} = \left(\frac{P_{sample} - P_{blank}}{P_{std} - P_{blank}}\right) \cdot F \cdot \frac{c_{std}}{m_{sample}}$$

c_{sample} – concentration of mercury in the sample (ng/mL or ng/g)
c_{std} – concentration of mercury in the standard (ng/mL); for example 10 ng/mL
P_{sample} – peak height in mm for the digested sample (for 5 mL taken from the 50 mL of the digested sample)
P_{std} – peak height in mm for the standard solution (1 mL of standard solution of 10 ng/mL was prepared in the same way as the sample and 5 mL out of 50 mL of that sample was taken for the measurement)
P_{blank} – peak height in mm of the blank test solution
F – dilution factor of the standard (for the case study above the dilution factor was 0.1; 1 mL of the standard solution 10 ng/mL was diluted to 50 mL and 5 mL was taken for the measurement)
m_{sample} – mass of the sample in g or mL

2.8. Quality control

Two reference materials were used in developing this SOP: Clin Chek 8847 (Recipe, Germany) and Seronorm trace elements urine – blank (Sero As, Norway). The analysts must obtain reference materials certified for mercury in urine at the concentrations typical for the

concentration range measured in the sample.

Each sample should be analysed in duplicates. If the parallel analyses differ by more than 10%, the sample needs to be reanalysed.

In each set of the analysis, the three blanks and duplicates of the quality control material (preferably reference material) need to be analysed and the quality control charts prepared (Fig. 4).

Fig. 4. Quality Control Chart (ClinChek 8847)
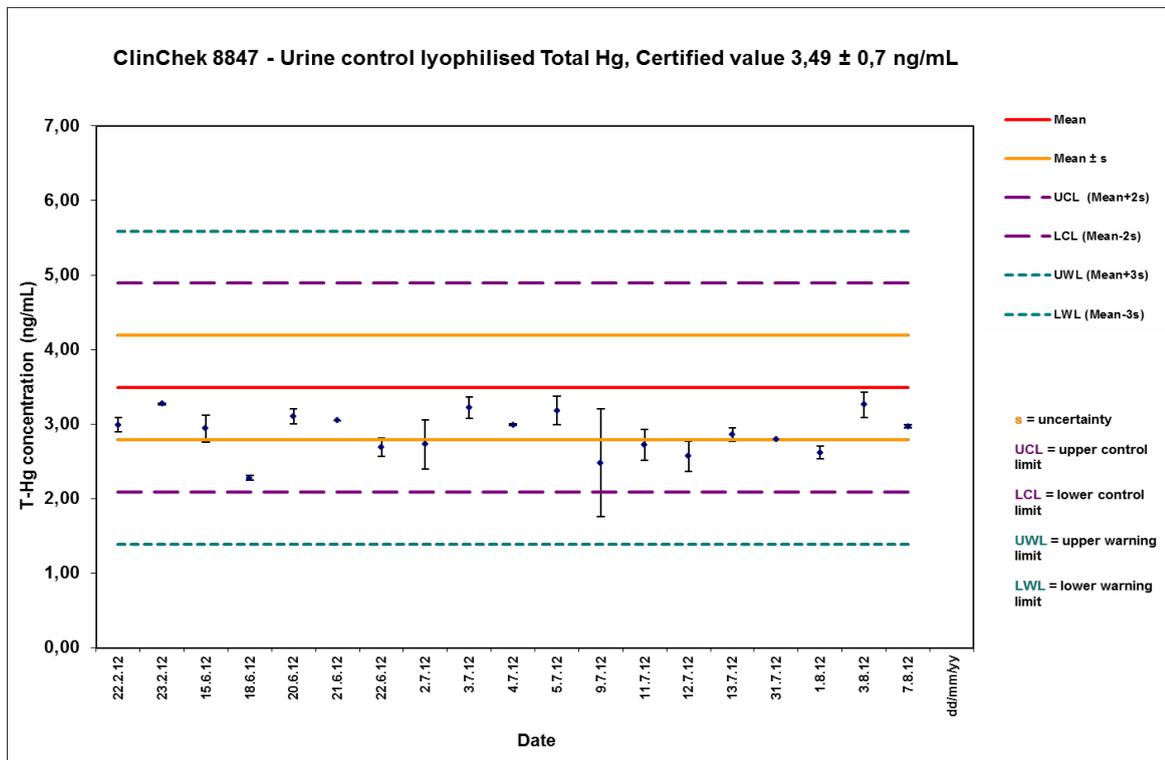
2.9. Evaluation of the method

Each laboratory should comply with the standard "ISO/IEC 17025:2005 General requirements for the competence of testing and calibration laboratories" *(15)*. The method should be validated for its performance criteria (sensitivity, linearity, recovery, robustness, precision, accuracy, LOD, etc.) and should be accompanied by measurement uncertainty estimation, as the latter is a fundamental property of a result and a requirement of the standard ISO/IEC 17025:2005. The concentration levels of mercury in urine are low and the LOD of the method should be at least 0.05 ng/mL, and the LOQ at least 0.1 ng/mL, to be able to measure concentrations in the general population.

For the method described in this SOP, the performance criteria and measurement uncertainty estimation are specified below.

2.9.1. Limit of detection and limit of quantification

The LOD was determined by assessment of mercury in 10 blank solutions. The concentration of mercury in 50 mL of the blank solution was 0.10 ± 0.010 ng. LOD was calculated using the following equation.

$$LOD = 3 \cdot SD_{blank}$$

SD – standard deviation

The LOD for the sample was then calculated as follows.

$$LOD = \frac{3 \cdot SD_{blank}}{V_{sample}(m_{sample})}$$

V_{sample} – volume (mL) or a mass (g) of the sample
m_{sample} – mass of the sample in g or mL

In the above case, the LOD was 0.03 ng/50 mL and the LOQ for the 2 mL of sample intake was 0.015 ng/mL.

The LOQ was calculated as five times the LOD.

$$LOQ = 5 \cdot LOD$$

The LOQ for the example above is 0.075 ng/mL.

2.9.2. Precision

As a measure of the degree of reproducibility of the described analytical method, routine analysis of urine samples over the course of a longer time period (e.g. one year) is used. For the purpose of demonstration, the results of one measurement series (n=15) of total mercury in urine are shown in Table 2. Each sample was analysed in 2 replicates.

Table 2. Results of duplicate measurements of total mercury in urine samples and their relative differences

Sample	Result D1 (ng/mL)	Result D2 (ng/mL)	Mean value (D1+D2/2)	Difference (D1-D2)	Relative difference (D1-D2/mean)
Urine 1	2.02	1.62	1.82	0.40	0.22
Urine 2	0.71	0.63	0.67	0.08	0.12
Urine 3	0.51	0.51	0.51	0.00	0.00
Urine 4	0.54	0.51	0.53	0.03	0.06
Urine 5	1.19	1.27	1.23	-0.08	-0.07
Urine 6	0.67	0.67	0.67	0.00	0.00
Urine 7	1.66	1.62	1.64	0.04	0.02
Urine 8	3.80	3.76	3.78	0.04	0.01
Urine 9	0.59	0.55	0.57	0.04	0.07
Urine 10	0.61	0.69	0.65	-0.08	-0.12
Urine 11	0.69	0.69	0.69	0.00	0.00
Urine 12	0.61	0.55	0.58	0.06	0.10
Urine 13	0.92	0.98	0.95	-0.06	-0.06
Urine 14	0.72	0.70	0.71	0.02	0.03
Urine 15	0.79	0.74	0.77	0.05	0.07

D1 = measurement 1; D2 = measurement 2.

To assess reproducibility or repeatability, standard deviation of replicate measurements is calculated using the following equation.

$$RSD_d = \frac{s_d}{\sqrt{n}}$$

RSD_d – relative standard deviation of duplicate measurements
s_d – standard deviation of relative differences (($D1$-$D2$)/*mean*)
n – number of replicates (n=2)

The repeatability calculated for the given set of measurements was 5.9%.

2.9.3. Trueness

The trueness of our results was estimated using the reference material ClinChek Urine Controls (Level I). As a measure of trueness of our results, recovery (*R*) was calculated based on measurements of the reference material over the course of six months. The observed levels were compared against the reference value using the following equation.

$$R = \frac{observed\ value}{reference\ value}$$

R – recovery

An example of measurements of total mercury in the reference material is given in Table 3.

Table 3. Measurements of total mercury in ClinChek Urine Controls (Level I)

Date	Mean value (ng/mL)	Reference value (ng/mL)	Recovery (%)
date 1	2.99	3.49	86
date 2	3.27	3.49	94
date 3	2.94	3.49	84
date 4	2.28	3.49	65
date 5	3.10	3.49	89
date 6	3.05	3.49	87
date 7	2.69	3.49	77
date 8	2.73	3.49	78
date 9	3.22	3.49	92
date 10	2.99	3.49	86
date 11	3.18	3.49	91
date 12	2.72	3.49	78
date 13	2.57	3.49	74
date 14	2.86	3.49	82
date 15	2.80	3.49	80
date 16	2.62	3.49	75
date 17	3.26	3.49	93
date 18	2.97	3.49	85

Based on the measurements given in Table 3, the recovery calculated was 83%.

2.9.4. Measurement uncertainty

Measurement uncertainty for total mercury in urine by acid digestion and CVAAS was estimated based on the approach and validation data set out in the ISO *Guide to the expression of uncertainty in measurement*. The procedure is described in the EURACHEM/CITAC guide, *Quantifying uncertainty in analytical measurement (16)*.

Step 1. The measurand was specified using the quantitative expression relating the value of the measurand to the parameters on which it depends (described in Section 2.7.).

$$c_{sample} = \left(\frac{P_{sample} - P_{blank}}{P_{std} - P_{blank}}\right) \cdot F \cdot \frac{c_{std}}{m_{sample}}$$

Step 2. Based on the quantitative expression, uncertainty sources were identified. These included the parameters listed in Table 4.

Table 4. Uncertainty components for the total mercury in urine

Input parameter	Value	Standard uncertainty	Relative standard uncertainty (%)
Sample signal (P_{sample})	30.0 mm	0.5 mm	1.6
Sample mass (m_{sample})	20 mg	0.06 mg	0.29
Volume of a sample in volumetric flask (V_{tot})	50 mL	0.12 mL	0.24
Volume of a sample aliquot analysed ($V_{analysed}$)	5 mL	0.0095 mL	0.2
Concentration of standard solution (c_{std})	10 ng/mL	0.014 ng/mL	0.14
Volume of standard solution (V_{std})	0.1000 mL	0.00094 mL	0.94

Step 3. In this step, uncertainty components were quantified. All uncertainty contributions must be expressed as standard uncertainties, that is, as standard deviations.

Standard uncertainties for the components identified from the quantitative expression were obtained from experimental data (e.g. pipette volumes) or from the producer's certificate (e.g. mass balance, volumetric flask).

The estimated standard uncertainties are given in Table 4. Relative standard uncertainties that do not exceed 10% of the largest uncertainty contribution are not taken into account in measurement uncertainty estimation. Among the listed uncertainties, uncertainties arising from the peak height (u_P) and the volume of standard solution (U_{Vstd}) were identified as significant.

Additional uncertainty components were estimated using validation data. For this purpose, reproducibility (repeatability) and recovery data was used.

The uncertainty of repeatability (u_{rep}) was 5.9% (Section 2.9.2), while the uncertainty of recovery ($u(Rm)$) was 8.5% and was calculated using the following equation.

$$u(\overline{R}_m) = \overline{R}_m \times \sqrt{\left(\frac{s_{obs}^2}{n \cdot \overline{C}_{obs}^2}\right) + \left(\frac{u(C_{ref})}{C_{ref}}\right)^2}$$

R_m – recovery
s_{obs} – standard deviation of the observed data
C_{obs} – mean value of the observed data
C_{ref} – reference value
$u(C_{ref})$ – uncertainty of reference value

In the final step, Step 4, combined uncertainty was calculated. Before combination, all uncertainty contributions must be expressed as standard uncertainties (standard deviations). The combined uncertainty (u_c) was calculated using the following equation.

$$u_c = \sqrt{u_P^2 + u_{Vstd}^2 + u_{rep}^2 + u_{rec}^2}$$

u_c – combined uncertainty
u_P – error due to repetitions of measures
u_{Vstd} – error due to the standards
u_{rep} – error due to reproducibility
u_{rec} – error due to recovery

Expanded uncertainty (*U*) was expressed by multiplying u_c with the factor *k*. The choice of the factor *k* is based on the level of confidence desired. For an approximate level of confidence of 95%, *k* is 2.

The estimated measurement uncertainty for the determination of total mercury in urine by acid digestion and CVAAS is 11%, expanded uncertainty (k=2) is 22%. The estimation is valid for a "normal" exposure range, that is below 5 ng/mL.

3. Analysis of creatinine in urine

The concentration of mercury and other chemicals in urine can vary significantly due to the amount of dilution with water, tests for contaminants in urine are often expressed in micrograms of contaminant per gram of creatinine (3). Creatinine is a by-product of protein metabolism in the muscles that is formed from creatinine phosphate. Creatinine mainly undergoes glomerular filtration in the kidney and is almost completely excreted. On average, adults of a normal body weight, aged 30–60 years excrete 1.0–1.6 g of creatinine per day.

The physiological formation of creatinine in healthy people is essentially proportional to their muscle mass, thus explaining why creatinine excretion is generally lower in women than in men. Children exhibit a daily excretion of creatinine that is quite strongly age-dependent. In addition to age and gender, creatinine excretion is also particularly influenced by the consumption of meat and the intake of certain medications, such as opiates and diuretics. Urine production can vary widely depending on the intake or loss of fluids and the consumption of coffee, alcohol or medications. In contrast, creatinine excretion generally remains relatively constant throughout the day, with only slight diurnal fluctuations. For this reason, the creatinine concentration in urine often serves as a reference value for the analysis of materials and their metabolites in urine. Thus, diurnal variations in the dilution of urine can be compensated for when exposure to xenobiotics is assessed. However, linking the concentration of hazardous substances in urine to creatinine concentration does not make sense in every case, and the above-mentioned factors that influence creatinine excretion also have to be taken into consideration.

If xenobiotics are reabsorbed to a significant extent in the tubular region of the kidneys, their concentrations cannot be assumed to be directly proportional to that of creatinine (17,18). Likewise, the use of creatinine content as a reference value for highly diluted or very concentrated urine samples also leads to invalid values for substance concentrations.

Thus, on principle, the Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area advises against calculating the concentration of hazardous substances or metabolites in urine with respect to creatinine concentration.

Despite this, the creatinine concentration should be measured in each urine sample that is to be tested for hazardous substances to help assess the results obtained. In the case of creatinine concentrations of less than 0.5 g/L or more than 2.5 g/L, the results obtained for the hazardous substances or their metabolites should not be taken into account in the reported findings (17).

3.1. Scope of the method

The method described herein is based on the Jaffé reaction (19) and permits the determination of creatinine in urine on a miniaturized scale using a photometric microplate reader. This method is used to achieve rapid and accurate quantification of creatinine in urine. The test range of the method is > 0.004 mg creatinine/mL urine.

The main advantage of the method described herein lies in the high number of samples that can be determined in a very short time. Moreover, it is easier to keep the reaction conditions stable as all samples are analysed at the end point of the reaction, thereby minimizing the risk of temporary measurement fluctuations.

3.2. Technical principle

Urine samples that have been diluted in a 1:50 ratio are applied to a microtitre plate and picric acid and sodium hydroxide are added. After a reaction time of 30 minutes, the absorbance of the reaction product is measured at an absorbance maximum of 500 nm using a photometric microplate reader.

Calibration is carried out using aqueous creatinine standard solutions, which are treated in the same manner as the samples by adding picric acid and sodium hydroxide solution and measured by photometry.

3.3. Safety precautions

The following safety precautions should be taken when analysing creatinine in urine.

- Safety precautions for biological hazards should be taken when working with urine.
- A biological safety cabinet should be used when diluting urine samples.
- Gloves, a laboratory coat and safety glasses should be worn when handling all solutions.
- Appropriate containers should be used for waste and biological residues. Pipette tips, autosampler tubes, gloves and other items that come into contact with urine should be placed in a biohazard autoclave bag or container.

3.4. Equipment, materials and solutions

3.4.1. Equipment

The following equipment is required for analysing creatinine in urine:

- vortex mixer used for vortexing urine specimens before removing an aliquot for analysis;
- micropipette 10–100 μL;
- micropipette 100–1000 μL;
- micropipette 1–10 mL;
- multichannel pipette 50–300 μL;
- analytical balance (readability 0.01 mL);
- centrifuge;
- microplate shaker;
- spectrophotometer;
- freezer (for long-term storage of samples and reagents);
- refrigerator (for intermediate storage of stock standards and reagents);

- water purification system (for ultrapure bidistilled water used in reagent and dilution preparation) – this equipment produces deionized water to > 18 MΩ•cm.

3.4.2. Materials

The following materials are required for analysing creatinine in urine:

- gloves (powder-free, low particulate nitrile or latex gloves)
- pipette tips (1000 μL, 100 μL and 10 mL)
- 96-well microplates
- 1, 5, 10 and 50 mL polypropylene test tubes.

3.4.3. Reagents and chemicals

The following reagents and chemicals are required for analysing creatinine in urine:

- ultrapure water
- picric acid, 1.2% solution (Panreac)
- sodium hydroxide (analytical grade)
- hydrochloric acid, 37%.

3.4.4. Reference materials

The following reference materials are used for analysing creatinine in urine:

- Creatinine SRM 914a (National Institute of Standards and Technology)
- Quality-control solutions URN ASY CONTROL levels 2 and 3 (Randox Laboratories).

3.4.5. Solutions

The following solutions are required for analysing creatinine in urine.

- 0.1 M hydrochloric acid: 871 μL of 37% hydrochloric acid is transferred to a 100 mL volumetric flask. The flask is subsequently filled to its nominal volume with ultrapure water.
- 0.3 M sodium hydroxide: 3 g sodium hydroxide is weighed and dissolved in approximately 100 mL ultrapure water. The solution is transferred to a 250 mL volumetric flask, which is subsequently filled to its nominal volume with ultrapure water.
- Picric acid working solution: 10 mL of 1.2% picric acid solution and 10 mL of 0.3 M sodium hydroxide are transferred to a 50 mL polypropylene tube. The working solution must be prepared freshly and protected from light.

3.4.6. Calibration standards

The following calibration standards should be used for this SOP.

- Creatinine stock solution (1g/L): 10 mg of Creatinine SRM 914a is weighed into a 10 mL volumetric flask. The flask is subsequently filled to its nominal volume with 0.1 M hydrochloric acid. The stock solution is stored at 4 ºC and its shelf life is two months.
- Calibration standards: the creatinine stock solution is diluted with ultrapure water in 10 mL volumetric flasks according to the scheme set out in Table 5. The calibration standards are stored at 4 ºC and their shelf life is one week.

Table 5. Volume and concentrations for preparation of calibration standards

Volume of stock solution of creatinine (µL)	Final volume of calibration standard (mL)	Concentration of calibration standard (g/L)	Equivalent concentration in the urine samples (g/L)
40	10	0.004	0.2
80	10	0.008	0.4
200	10	0.020	1.0
400	10	0.040	2.0
800	10	0.080	4.0

3.5. Sample treatment and preparation

Powder-free gloves must be worn during sample handling.

The samples to be analysed are removed from the freezer and allowed to warm to room temperature. They are then vortexed and centrifuged at 3000 rpm for two minutes. Two commercial quality urine control samples of different concentrations (Assayed Urine Chemistry Control Level 2 and Level 3) are included in each analytical series. Each vial is reconstituted with 10 mL of bidistilled water and left to stand for 30 minutes at room temperature before use. It can be aliquoted and stored at -20 °C for two weeks.

Samples and quality controls diluted at 1/50 are prepared in triplicate. Next, 20 µL of sample or quality control is pipetted into 1.5 mL tubes. Then, 980 µL of ultrapure water is added. The tubes are capped and shaken to homogenize the dilution.

Next, 25 µL of standards, diluted samples and controls is added to each well of a 96-well microplate, according to the distribution outlined in Table 6.

The plate is covered with a lid and shaken on an orbital shaker at room temperature protected from light. After 30 minutes the plate is read at 492 nm.

Table 6. Distribution of standards, samples and controls on the microplate

	1	2	3	4	5	6	7	8	9	10	11	12
A	water	water	water	P1	P1	P1	P2	P2	P2	P3	P3	P3
B	P4	P4	P4	P5	P5	P5	S1	S1	S1	S2	S2	S2
C	S3	S3	S3	S4	S4	S4	S5	S5	S5	S6	S6	S6
D	S7	S7	S7	S8	S8	S8	S9	S9	S9	S10	S10	S10
E	S11	S11	S11	S12	S12	S12	S13	S13	S13	S14	S14	S14
F	S15	S15	S15	S16	S16	S16	S17	S17	S17	S18	S18	S18
G	S19	S19	S19	S20	S20	S20	S21	S21	S21	S22	S22	S22
H	S23	S23	S23	S24	S24	S24	C1	C1	C1	C2	C2	C2

Notes:
P1: aqueous 0.004 mg/mL creatinine standard
P2: aqueous 0.008 mg/mL creatinine standard
P3: aqueous 0.2 mg/mL creatinine standard
P4: aqueous 0.4 mg/mL creatinine standard
P5: aqueous 0.8 mg/mL creatinine standard
C1: 1/50 dilution quality control URN ASY CONTROL 2
C2: 1/50 dilution quality control URN ASY CONTROL 3
S1–S24: 1/50 sample dilutions

3.6. Procedure

3.6.1. Preparation of analytical equipment

Turn on the spectrophotometer.

The system requires approximately 15 minutes of pre-heating time before measurements can be started.

3.6.2. Sample measurement

Measure the samples prepared according to point 7.1 at 500 nm

3.6.3. Calculation of the analytical results

Data are reported directly by the equipment in terms of mg of creatinine/mL by interpolating the reading against the calibration curve taking into account the sample dilution.

The final value reported corresponds to the average of the three replicated measurements per sample. The standard deviation of these measurements can be calculated according to the following formula.

$$SD = \sqrt{\frac{\sum (c_i - \bar{c})^2}{n - 1}}$$

SD – standard deviation
c_i – individual value
c – mean
n – number of determinations

3.6.4. Reportable results range

It must be checked that the values obtained for the quality controls meet the acceptance criteria established in their certificates of analysis. If these criteria are not met, analysis should be performed again.

The relative standard deviation of the three measurements for a sample should not be higher than 5%. Otherwise, the Grubbs test should be applied to determine whether one of the values is a significant outlier.

$$Z = \frac{|\text{Mean} - \text{Suspected value}|}{SD}$$

Z – Z value (for evaluation according to Grubbs test)
SD – standard deviation

If Z is greater than 1.15 this value can be rejected and the concentration of the sample calculated as the mean of the two remaining values. Otherwise, the sample should be retested.

Creatinine values are reportable in the range 0.3–3 mg/mL.

3.7. Quality control

The precision and accuracy of biomarker analyses carried out by toxicological laboratories must be continuously checked by means of quality assurance procedures.

In general, quality assurance in medical laboratories comprises both internal and external quality control, which is described in detail in the *Quality control programme for mercury human biomonitoring*.

3.7.1. Internal quality control

Internal quality assurance serves to systematically monitor repeatability in order to detect random errors and ensure the accuracy of quantitative laboratory investigations.

In practice, the repeatability is controlled by using a control material (reference material), which is measured as part of each analytical series. The results of daily or batch-wise internal quality control are entered into control charts.

If not commercially available, a control material (reference material) can be prepared by spiking a pool of native biological material (blood, urine, etc.) with a defined amount of the analyte (biomarker). Aliquots from this pool can be used for internal quality control as well as for inter-laboratory comparison programmes. These aliquots have proven to be, and to remain, homogeneous under specified storage and shipment conditions, with the analyte concentration remaining unchanged. A control material should cover the whole concentration range (e.g. Q_{low}, Q_{medium}, Q_{high}), as well as blanks.

Accuracy should preferably be tested using a certified reference material, which is a (biological) material containing a certified concentration of one or more analytes. Certification is performed within a programme in which laboratories that are highly skilled in analysis of the biomarker in question, analyse the control material.

A certified value is established for each analyte following a validation procedure that includes expert judgment as well as statistical procedures. Certified reference materials are therefore expensive and should only be used when validating or revalidating an analytical method.

3.7.2. External quality control

External quality control is a means of improving the comparability and accuracy of analytical results. Comparability is the pre-state of accuracy and ensures that analytical results can be compared between laboratories and with the corresponding limit values.

Comparable and accurate results in HBM are necessary to achieve equal heath prevention irrespective of the laboratory that analyses the biological sample.

An inter-laboratory comparability investigation (ICI) is a means of harmonizing analytical methods and their application, thereby improving the comparability of analytical results.

Control materials (reference materials) can be used for this purpose. ICIs are even necessary when laboratories use the same analytical SOP.

An external quality assessment scheme (EQUAS) is a means of improving the accuracy of analytical results. For this purpose, a control material is usually analysed in reference laboratories that have been shown to be highly skilled in analysing a specific biomarker. The results obtained by the reference laboratories form the basis on which the assigned values and tolerance ranges for each of the biomarkers tested are determined. Those laboratories that participate in an EQUAS are certified for those results that fall within the tolerance ranges.

For this SOP, quality control materials are used to evaluate the accuracy and precision of the analysis process and to determine whether the analytical system produces results that are acceptably accurate and precise.

Quality control of the analytical results is carried out using the reference materials URN ASY CONTROL 2 and 3 (Randox).

It should be checked that the values obtained for quality controls meet the acceptance criteria established in their certificates of analysis. If this criterion is not met, analysis should be performed again.

Only those measurements performed between two quality controls whose values lie within the established range (assigned value of the reference material ± uncertainty in that level) are considered valid.

External quality controls are performed by participation in round-robin experiments. As an example, it is recommended to participate regularly in a G-EQUAS test organized by the Institute and Out-Patient Clinic for Occupational, Social and Environmental Medicine of the Friedrich-Alexander-University Erlangen-Nürnberg, Germany.

3.8. Evaluation of the method

3.8.1. Linearity

The linearity of an analytical procedure is the ability (within a set range) to obtain results which are directly proportional to the concentration of analyte in the sample. This parameter is evaluated by studying increasing analyte concentrations. In this SOP, the linearity of the method has been tested in the range 0.004–0.08 mg/mL creatinine.

The data obtained are analysed statistically to obtain the regression curve, correlation coefficient, determination coefficient and coefficient of linearity. A linear curve with a determination coefficient higher than 0.999 should be obtained.

3.8.2. Precision

This is a measure of how much the analytical results are scattered due to random errors. Precision is described statistically by means of the standard deviation or confidence interval. We can distinguish between the following:

- precision under repeated conditions (repeatability)
- precision under comparable conditions (reproducibility).

Samples in the range 0.2–3 mg/mL were used to determine the precision. Table 7 shows the results obtained for repeatability and reproducibility.

Table 7. Maximum standard deviation allowed

Concentration (mg/mL)	RSD_{repet}	RSD_{reprod}
0.2	11.5	7.3
0.4	4.8	5.4
0.7	2.2	4.7
2.1	1.8	2.5
2.5	4.8	5.0

RSD_{repet} = relative standard deviation for repeatability;
RSD_{reprod} = relative standard deviation for reproducibility.

3.8.3. Accuracy

This is a measure of the deviation of the measured value from the correct ("true") value due to a systematic error. The following approaches can be used to test the accuracy of a method:

- performance of recovery tests (spiking procedures);
- participation in inter-laboratory comparability investigations in which the theoretical value is ascertained by authorized reference laboratories;
- comparison of the analytical procedure to be validated with a reference procedure certified for determination of the parameter in the relevant sample matrix;
- comparison of the analytical results for a certified reference material with the certified reference value.

The accuracy was determined by adding known amounts of creatinine to the samples used for the determination of precision. Mean recovery rates obtained were in the range 98.2–104.4%.

The lower LOQ indicates the lowest possible analyte concentration that can be determined with a pre-defined uncertainty (usually 33%). The upper limit of quantification indicates the highest possible analyte concentration that can be determined.

The LOQ must be included in the calibration curve and can be calculated using various different methods.

Determination of the signal/background noise ratio
The background noise is determined as follows.

- The intensity of the background noise (s_0) is determined in relation to the analyte.
- The LOD is calculated as three times the mean intensity of the background noise signal (LOD = 3 x s_0).
- The LOQ is calculated as nine times the mean intensity of the background noise signal (LOQ = 9 x s_0).

Other procedures
It should be noted that blank values in native samples have an influence on the choice of method and the approach used:

- standard deviation procedure (according to EURACHEM)
- blank value procedure (according to DIN 32 645)
- calibration curve procedure (according to DIN 32 645).

In this SOP, the LOQ has been calculated using the calibration curve procedure and the result obtained corresponds to the lowest value of the calibration curve, 0.004 mg/mL creatinine.

3.9. Sources of error

This analytical method is based on the Jaffé colour reaction *(19)* in which the active methylene

group of creatinine reacts with the C3 atom of picric acid *(20,21)* to form a coloured reaction product *(19,21)*. However, this colour reaction between picric acid and creatinine is not specific to this substance. As a general rule, reducing compounds or compounds with a methylene group activated by -NO2, -CONH2, -CH2=CH2-, -COOR or -N=N-, can also form coloured products. Thus, no interfering reactions are caused by glucose, fructose, maltose, hydroxylamine or ascorbic acid, whereas aminoacetone, γ-aminolevulinic acid and aminooxyacetic acid exhibit a colour reaction with picric acid *(22)*.

As the concentrations of the above-mentioned chromogens are very low in urine *(20)*, interference caused by them can be regarded as insignificant. Thus, for example, the concentration of γ-aminolevulinic acid in urine is some 100–1000 times lower than that of creatinine.

Picric acid solution is sensitive to light and should therefore be kept in the dark. This also applies to the prepared microtitre plate during incubation.

When working with microtitre plates, it is essential to ensure that no liquid splashes out of the wells while pipetting, thus leading to contamination of other samples. To prevent this, use of a freely moving hand dispenser is recommended when pipetting the picric acid working solution.

Soiling (e.g. fingerprints) on the underside of the microtitre plate may lead to considerable interference during measurement. In extreme cases this may render the plate unreadable for the measurement device. As a result, the underside of the plate must be kept clean and should be wiped with a cloth soaked in ethanol before measurement.

3.10. Alternative method: determination of specific gravity in urine samples

As an alternative to creatinine analysis, specific gravity (SG) can be determined in urine samples to normalize the urine mercury levels for inter-individual differences in urine dilution *(23)*. This method has been widely used in many HBM studies. SG is determined in a drop of urine using a refractometer. This is a simple hand-held instrument, which is very easy to operate. The following procedure applies to the PAL-10S refractometer (Atago, Japan) but any similar instrument can be used.

The equipment required is as follows:

- refractometer PAL-10S, Atago, Japan
- containers for urine collection (can be the same as for mercury analysis)
- pipette (0.1–1 mL)
- distilled water
- cleaning cloth or disposable tissues
- gloves.

The procedure for determination of specific gravity in urine samples is as follows.

1. The temperature of the distilled water used for calibration (zero setting) and the sample should be the same as the ambient temperature.

2. Calibrate the refractometer by placing distilled water (approximately 0.3 mL) onto the prism surface and press the START key. If the display indicates "1.000", zero setting does not need to be performed. If the indicated value is not "1.000", press the ZERO key with the water left on the prism. After "000" is displayed, zero setting has been successfully completed. Remove the water from the prism surface using a soft non-abrasive tissue. This should be done before you begin testing, and after every 10 samples or so to ensure that the calibration remains accurate.
3. Measurement. Clean the surface with distilled water and dry with a soft non-abrasive tissue. Place a drop of urine (approximately 0.3 mL) onto the prism surface. Press the START key. The measurement value will be displayed on the screen. Remove the sample by wiping it off with a soft tissue. Use distilled water to remove any remaining sample. Dry off any excess moisture with a clean, dry tissue. To turn off the display, press and hold down the START key for approximately two seconds.
4. Calculation. As a standard in SG normalization calculations, SG means of 1.013 for females and 1.019 for males are normally used; calculation is described in the literature *(24)*.

$$U_{biomarker}/SG = U_{biomarker} * \frac{(SG_{std} - 1)}{(SG_{ob} - 1)}$$

$U_{biomarker}$ – level of a substance (e.g. mercury) measured in urine
SG_{ob} – specific gravity, observed
SG_{std} – mean specific gravity in a studied population

The SG results will normally range from 1.000 (which is equivalent to water) up to 1.035 (very dehydrated), but can also reach higher levels.

4. Interpretation of results

Urine mercury levels are usually considered the best measure of recent exposures to inorganic mercury or elemental mercury vapours because urinary mercury is thought to indicate most closely the mercury levels present in the kidneys *(25)*. However, inorganic mercury accumulates in the kidney and is slowly excreted through the urine. Therefore urine mercury levels can also represent exposures to elemental mercury and/or inorganic mercury that occurred sometime in the past *(3)*.

A strong correlation between elemental mercury levels in inhaled air and levels in urine, at medium and high concentrations, has been reported. The maximum urine mercury concentration set by WHO *(26)* is 50 µg/g creatinine. Mercury urine levels rarely exceed 5 µg/g creatinine in people who are not occupationally exposed to mercury *(3)*.

Mercury levels exceeding 20 µg/L urine have been found in urine samples from miners who frequently burn gold-mercury amalgams in open pans. Very high mercury concentrations in urine (as high as 1168 µg/L) were reported in workers of gold shops in Amazonian villages. The gold shop workers (who work in confined environments) had higher concentrations of mercury in urine than miners burning amalgam outdoors. In Alta Floresta, Mato Grosso, Brazil, the urine of employees in gold shops (where gold was melted in fume hoods with no filters) was analysed; the results showed mercury urine levels greater than 20 µg/L for at least 13 of 17 workers sampled *(27)*.

The German Human Biomonitoring Commission's reference value for adults without dental amalgam fillings is 1 µg/L in urine *(28)*. The corresponding reference value for children without amalgam fillings is 0.4 µg/L *(28)*. The health-based HBM-I guidance value for mercury in urine is 7 µg/L or 5 µg/g creatinine. Geometric mean levels in adults in most countries are below the reference value *(8)*.

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Annex 1. Urine sampling instructions for participants

Pleased read these instructions carefully before taking the first morning urine sample.

Note. At least five hours should have passed since last urination.

1. Go inside the toilet.
2. Wash your hands with soap and water and then dry them.
3. Remove the urine vessel from the zip-lock plastic bag. (Please only use the provided vessel. This vessel was pre-treated for this study.)
4. Open the urine vessel by unscrewing the lid.
5. Discharge your first morning urine into the vessel until it is filled to the pre-marked line.
6. Screw the vessel lid on tightly.
7. Place the urine vessel back into the zip-lock plastic bag.
8. Keep the sample at 4–8 ℃ until you give it to the health-care staff (no longer than 24 hours).

Thank you very much for your cooperation.

Annex 2. Questionnaire for urine sample collection

Name of mother		
Medical record number		
ID of mother		
Medical worker or survey technician	_____ _____ Signature Printed name	
1	Is this a sample of urine taken in the morning?	❑ Yes ❑ No
2	Date and time of sample collection	------/-----/-------/ -------/------ day/month/year hour/min
3	How many hours ago did you last urinate prior to this sample collection?	hours
4	How many hours prior to this sample collection was your last meal?	hours
5	Have you been in the presence of anyone smoking indoors in the last 24 hours?	❑ Yes ❑ No

6	Which of the following processed foods have you eaten in the last 24 hours? Check all that apply.	❑ Frozen foods packaged in cardboard ❑ Frozen foods packaged in polyethylene ❑ Fast foods ❑ Canned foods ❑ Dried foods packaged in a plastic container ❑ Dried foods packaged in polyethylene
7	When was the last time you had fish or other types of seafood prior to this sample collection?	❑ Today ❑ Yesterday ❑ Day before yesterday

Annex 3. Sample reception list

	Urine			Aliquoting urine samples	
	U1	**U2**	**X U**		
Volume	Mercury 5 mL	Creatinine 5 mL	Biobank x 40 mL or x 10 mL		
Store t° field work and transport	Cool box	Cool box	Cool box		
Store t° laboratory	-20 °C	-20 °C	-80 °C		
Identification number				Date	hour
ID					
ID					
ID					
….					