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Stabilization of urinary biogenic amines measured in clinical chemistry laboratories

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ABSTRACT

Urinary 5-hydroxyindoleacetic acid (5-HIAA), vanillylmandelic (VMA), homovanillic acid (HVA), catecholamines and metanephrines are produced in excess by catecholamine-producing tumors. These biogenic amines are unstable at low or high pH and require hydrochloric acid (HCl) to prevent their degradation. However, HCl addition may result in very low pH causing degradation or deconjugation of several metabolites. This study evaluated the buffering properties of sodium citrate to stabilize all biogenic amines.

The metabolite concentrations were measured by LC-MS/MS or by a coulometric assay in 22 urine samples collected native and with HCl or sodium citrate. We studied the effect of pH, time (48 h, four weeks) and storage temperature at 22 °C, 4 °C, and −20 °C.

We found that catecholamines degradation was prevented by HCl and citrate and that 5-HIAA was degraded in 5 out of 22 samples collected with HCl. All biogenic amines were efficiently stabilized by citrate for four weeks at 22 °C, except epinephrine (48 h at 4 °C, or four weeks at −20 °C). Sodium citrate did not cause quantification or analytical artefacts concerns. In conclusion, sodium citrate is a non-hazardous alternative to HCl for patients to send unfrozen urine samples to the laboratory which may safely store the sample for four weeks.

1. Introduction

Carcinoid tumors are rare neuroendocrine tumors deriving from enterochromaffin cells secreting serotonin [1]. The measurement of urinary 5-hydroxyindoleacetic acid (5-HIAA), the main product of serotonin metabolism, is used for the diagnosis and follow-up for this tumor [2,3]. Neuroblastomas are neoplasms that arise from neural crest-derived cell precursors of postganglionic sympathetic neurons and are characterized by excessive production of catecholamines (CATs) [4,5]. The measurements of metanephrines (MNs), as well as vanillylmandelic acid (VMA) and homovanillic acid (HVA) in urine, end products of norepinephrine (NE) and dopamine (DA) metabolism, are used to help the clinician with diagnosis and follow-up for neuroblastomas [6]. Pheochromocytomas and paragangliomas are neuroendocrine tumors characterized by excessive production of CATs: epinephrine (EPI), NE, and DA [7]. The measurement of their respective O-methylated metabolites (MNs), metanephrine (MN), normetanephrine (NMN), and 3-methoxytyramine (MT), in urine or plasma, is recognized as the most sensitive and specific test for the diagnosis of pheochromocytomas [8]. All these biogenic amines may be determined either in 24-h urine collections or within single spot urine samples.

The stability of the biogenic amines in urine is key to providing a reliable diagnosis and has been the subject of discussion for years [9–13]. Today, urine collection with hydrochloric acid (HCl) is the most accepted method for all of these metabolites [13]. However, its use is regularly questioned because of the inability of this strong acid to stabilize the pH whereas 5-HIAA is unstable at very acidic pH [13,14], CATs are degraded at pH close to neutral pH and above or in extreme acid conditions [9,10,13], and sulfated MNs and CATs may be deconjugated at very low pH [11,13]. Therefore, the initial excess of HCl present in the container provided to the patient before collecting urine may contribute to 5-HIAA degradation and the deconjugation of sulfated catecholamines during the first urination. Moreover, HCl is a hazardous acid to handle for patients during urine collection.

Recently, Peitzsch et al. reported the efficient stabilization of free MNs and CATs in urine for seven days by adding sodium dihydrogen citrate (later referred to as “citrate”) [12]. Thanks to its buffering capacity, free MNs and CATs were not degraded in the ten studied citrate

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urines whose initial pH ranged from 5.5 to 8.7. However, this study did not include the other metabolites of endogenous bioamines often co-requested such as 5-HIAA, VMA, HVA and deconjugated (i.e., total) metanephrines. Mid-term stability (up to one month) was not evaluated although this corresponds to the time necessary for an additional analysis in a sample to be requested and carried out.

Our study aimed to evaluate the capacity of citrate to prevent the post-collection degradation of 5-HIAA, as it is unstable at low pH. We also extended the study to total MNs (the sum of free and deconjugated MNs) since they are more frequently used than free metanephrine by laboratories because IVD kits are available, and to VMA and HVA since their measurements are recommended with CATs for the biochemical diagnosis of neuroblastoma. We studied the stability of all these metabolites for 48 h and four weeks. The 48-hour period corresponds to the usually maximum time for the sample to reach the laboratory, and the four-week period to the longest storage period of primary samples in case of repetition or addition of measurements. We included a total of 22 urine samples to represent the diversity of urine composition, including four urines with pH below 6.0.

2. Materials and methods

2.1. Chemical and reagents

Hydrochloric acid 37% (HCl) and sodium hydroxide 10% (NaOH) were purchased from Merck (Darmstadt, Germany). Sodium dihydrogen citrate, purum, p.a. (CAS number 18996–35-5) was obtained from Sigma-Aldrich (Steinheim, Germany).

Urine spots were obtained from our laboratory (n = 22, aged 25 to 65, 50% males) and were aliquoted in 250 μL polypropylene tubes (Fig. S1). The pH of urines was immediately measured using pH indicator strips. Diluted NaOH solution was added to five of the 22 urines to reach a pH between 8.0 and 10.0. Each of the 22 urine samples was split into three sub-samples, one of which was supplemented by 5 mL of 6 M HCl per liter of urine ("HCl urine"), the second by 10 g citrate per liter of urine ("citrate urine"), and the third was unmodified ("native urine"). The sub-samples were then split into 18 aliquots and stored at either 22 °C, 4 °C, or −20 °C. After 48 h and four weeks, three aliquots of each storage and acid conditions were stored at −20 °C until analysis. Fig. S1 summarizes the sample handling.

2.2. Analytical methods

All the analytical methods used throughout this study were the routine methods from our laboratory and were fully validated. 5-HIAA, VMA, and HVA were measured in one aliquot by LC-MS/MS using a dilute-and-shoot sample preparation [15]. The total MNs were measured on the second aliquot subjected to acid hydrolysis [16] and analyzed by LC-MS/MS using a method adapted from Dunand et al. [17]. The CATs were quantified in the third aliquot using the RECIPE catecholamine kit (München, Germany) using a 5600A electrochemical Coularray system coupled to an isocratic HPLC (ESA-Dionex, Sunnyvale, CA, USA). CATs were also quantified by LC-MS/MS [18] in six additional urines to assess the compatibility of citrate with this method.

2.3. Data analysis

Data processing, statistical calculations, and graphical representations were prepared using Microsoft Excel 2016 (Redmond, WA, USA) and Prism 8.3.0 (GraphPad Software, San Diego, CA). The stability of the bioamines was described using two parameters: the mean recovery and the coefficient of variation (CV) calculated on the recoveries. The recovery was defined as the ratio of the concentration measured in the studied condition to the concentration measured after 48 h at −20 °C. The absence of analytical bias caused by the addition of the acids was assessed by paired Wilcoxon *t*-test p-values. The effect of citrate concentration on the analytes concentrations was evaluated by 2-way ANOVA using Prism.

3. Results

3.1. pH

The median pH of the fresh urines, before freezing, was 6.0, with 95% of the values between 4.8 and 7.5 (see Fig. 1 and Table S1). After a 48 h freeze–thaw cycle, the median pH increased by 0.5 unit (95% of the values between 5.3 and 9.0). The addition of HCl decreased the median pH to 3.8, with 95% of the values ranging between pH 1.0 and 7.0, and with five pH values below 2.0. The addition of citrate decreased the median pH to 4.5, with 95% of the values between pH 3.8 and 5.2.

3.2. 5-HIAA stability

Stability of 5-HIAA was excellent in native and citrate urines, with mean recovery values between 80% and 120%, as well as a CV below 20% in all studied conditions (a high CV calculated on the recoveries is an indicator of degradation) (Fig. 2 and Table S2). One data point is above 120% in each condition with citrate urine, originating from the same sample, without any known reason (pH = 4.0). We suspect an unexpected negative bias on the measurement of the reference value that resulted in overestimated values (120–150%) for the other conditions. Conversely, a clear degradation of 5-HIAA was observed in HCl urines after four weeks of storage at 22 °C, and for some urines at −20 °C and + 4 °C. The CV on recoveries was above 20% after four weeks of storage at 22 °C. The five HCl urines with a recovery below 80% presented a pH of 2.0 or below, and vice-versa (Fig. 3), as already reported elsewhere [13,14]. No analytical interference was observed in any of the LC-MS/MS chromatograms following the addition of citrate to the urines, despite the use of a dilute-and-shoot method [15]. The absolute concentrations of 5-HIAA in the samples were unchanged with the addition of citrate and HCl (p = 0.68 and 0.06, respectively) (Table S3).

3.3. VMA and HVA stability

VMA and HVA, which are measured by the same LC-MS/MS assay as 5-HIAA [15], were stable in all urine samples conditions including in the native urines (Fig. S2 and Table S4), as already reported elsewhere [13].

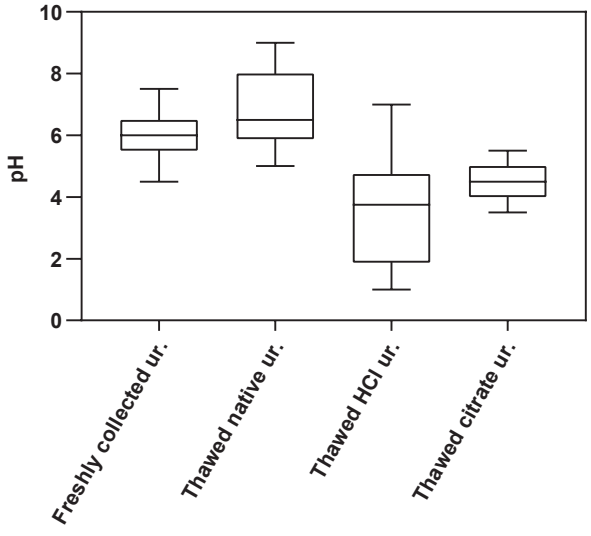

Fig. 1. pH measured in freshly collected urines before freezing (left plot) and in thawed native, HCl and sodium citrate urines (n = 22 each) prior to analysis. The center line of each box represents the median, and the whiskers show the 2.5th and 97.5th percentiles. Supplemental Table 1 displays the detailed values.

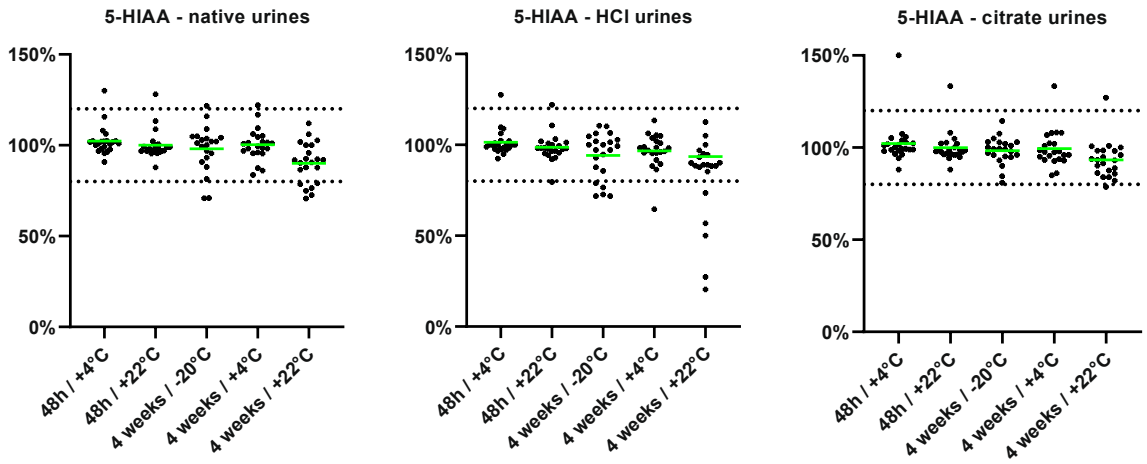

Fig. 2. stability of 5-hydroxyindoleacetic acid (5-HIAA) in native, HCl and sodium citrate urines (n = 22 each), reported as concentrations relative to the value measured after 48 h storage at −20 °C. The green line shows the mean and the dotted lines the 80% and 120% limits. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

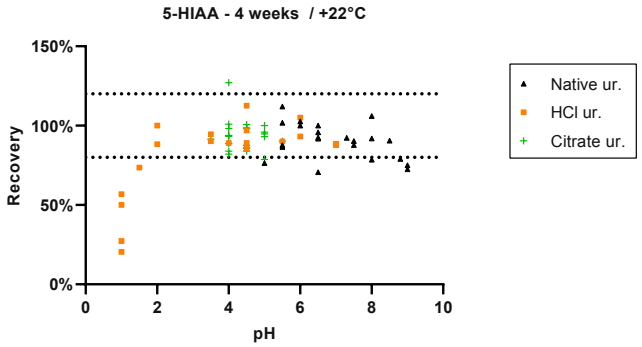

Fig. 3. Recoveries of 5-HIAA in native, HCl and sodium citrate urines (n = 22 each) as a function of the pH of the samples, stored four weeks at 22 °C.

No analytical interference was observed in any of the LC-MS/MS chromatograms following the addition of citrate to the urines. The absolute concentrations of VMA and HVA in the urine samples were unchanged with the addition of citrate ($p = 0.98$ and 0.32, respectively) and the concentration of VMA with the addition of HCl ($p = 0.56$). Conversely, the addition of HCl significantly changed HVA concentrations ($p = 0.04$) (Table S3).

3.4. Total metanephrines stability

The three metanephrines showed excellent stability in all studied conditions including in the native urines, with the exception of MT in native urines stored for four weeks, where the CV values between 18 and 23% indicate a slight degradation (Fig. S3 and Table S5). No analytical interference was observed in any of the LC-MS/MS chromatograms following the addition of citrate to the urines. The acid hydrolysis of sulfated MNs was not affected by the presence of citrate. The concentrations of the total MNs in the urine samples were unchanged with the addition of citrate and HCl ($p > 0.05$ for all conditions) (Table S3).

3.5. Catecholamines stability

In native urines, the three CATs suffered significant degradation. Recoveries were below 80% after 48 h of storage at 22 °C and after four weeks of storage at 4° and 22 °C, the CV on recoveries were > 20% for all but one conditions, and catecholamines were undetectable in at least one urine for each condition (Fig. S4 and Table S6). The addition of HCl or citrate significantly prevented degradation of NE and DA in all conditions (recoveries > 80% and CV < 20%), except four weeks at 22 °C with HCl (CV > 20%), and of EPI for 48 h at 4 °C and four weeks at −20 °C.

Fig. S5 displays recoveries of the analytes vs the pH of the urines after four weeks storage at 22 °C. Most of the urine samples presenting a relative recovery below 80% had a pH above 6.0 (native and paradoxically HCl for urines difficult to acidify). This is in line with previous stability studies [9–11,13] showing that the acidification of urines with HCl prevents the degradation of catecholamines. Conversely, the pH of all urines with a recovery of catecholamines above 120% was below 2.0, suggesting that hydrolysis of sulfonated catecholamines occurs in highly acidic environment [10,11].

The absolute concentrations of CATs in urine samples were unchanged with the addition of HCl ($p \geq 0.13$). Conversely, the addition of citrate significantly changed the concentrations of EPI, NE and DA concentrations ($p = 0.05$, 0.003 and < 0.0001, respectively) (Table S3), measured using our coulometric assay, which prevented the measurement of CATs in citrate urine. After the study was completed, a new LC-MS/MS assay [18] replaced the coulometric assay in our laboratory. Six additional urine samples analyzed by LC-MS/MS showed no difference between absolute concentrations of EPI, NE and DA in citrate vs native urines ($p = 0.56$, 0.50, 0.69, respectively) and the absence of interference in the chromatograms, as already demonstrated by Peitzsch et al. [12].

3.6. Citrate concentration

The compatibility of the assays with higher citrate concentrations than the recommended 10 g/L concentration was assessed in three additional urine samples (Fig. S6). Up to 100 g of citrate per liter of urine, the concentrations of all biogenic amines were unmodified ($p > 0.05$), except for the MNs in urines supplemented with 100 g/L of citrate, where MN, NMN, and MT concentrations were decreased by 25, 28 and 30% ($p = 0.0165$, 0.0075 and 0.015), respectively. As the stability of total MNs is not pH-dependent, this suggests that citrate interfered with MNs during extraction or analysis. No interfering peak was observed on any chromatogram when higher citrate concentrations were added. The pH of all urine samples was unmodified, except in one of the samples supplemented by 100 g/L of citrate, where the pH was lowered by 0.5 unit (Fig. S7).

4. Discussion

The purpose of this study was to solve the issues observed with the preanalytical stability of the most frequently measured urinary biogenic amines in clinical chemistry laboratories. Our aim was to standardize the stabilization method to prevent repeated urine collections and confusion due to preanalytical uncertainties when clinicians ask for the measurement of several biomarkers in the same sample.

A similar stability study has been performed with CATs and free MNs in urine, which focused on the short-term conditions of stability, and was not taking into consideration the other biogenic amines, precluding a global solution for analysts [12]. We included 5-HIAA, VMA and HVA in urine. 5-HIAA is a known unstable molecule used to exclude carcinoid tumors as recommended by the European Neuroendocrine Tumor Society (ENETS) [2] that is often requested together with metanephrines in urines. VMA and HVA are frequently requested with catecholamines as recommended for the diagnosis and follow-up of neuroblastoma [19]. We have deliberately chosen a study design with only two time-points to include a sufficient number of samples (twenty-two) to better cover the variability of pH and antioxidant components found in urines treated in a clinical laboratory. The first measurement was carried out after 48 h, which is the usual maximal time for a sample to be collected and delivered to the testing laboratory. A second quantification was established after four weeks, which is the usual maximal time for an additional analysis to be requested by the general practitioner. The starting concentration (100%) for each analyte was not established immediately after urine collection, but after a freeze–thaw cycle, in order to have a constant point of comparison for all samples, and to reproduce what usually occurs in laboratories.

Paired *t*-test and ANOVA were not suitable for highlighting clinically relevant change in concentrations in the different conditions, because the significant differences highlighted were mostly correlated with analytical precision and bias. For example, mean relative NE concentration measured after four weeks at 22 °C decreased by 6.7% with HCl, with a CV of 43.2%. In citrate urines, it decreased by 5.1% with a CV of 5.1%. The corresponding Wilcoxon p-values vs 48 h at −20 °C were 0.64 and 0.0005, respectively, although the second situation presents no clinically significant difference (see Fig. S4). Therefore, the stability of the biogenic amines was described by the mean recoveries, as already done previously [9,10,12], and by the coefficient of variation (CV) calculated on the relative recoveries, which is an excellent indicator of degradation.

We found that 5-HIAA was degraded at pH below 2.0, as reported earlier [13,14]. We also observed that catecholamines are degraded at pH above 6.0 and that their concentrations increased at very low pH due to sulfate hydrolysis, in agreement with previous findings [10,12,13]. This study showed that VMA, HVA, and total MNs are stable in all studied conditions. For decades, HCl addition has been widely accepted as the gold standard to maintain the stability of biogenic amines in urine [13] as it provides pH values of ca. 4 (median pH 3.8 in our study, n = 22). However, as HCl is a strong acid, the pH of urines supplemented with it may reach extreme values such as 1 or 7 depending on the buffering properties of the urine, resulting in the breakdown of 5-HIAA, the degradation of catecholamines, or the deconjugation of their sulfated forms. Our data demonstrate the superiority of using a weak acid buffer, such as citrate, resulting in a pH range of 3.8–5.2, rather than a strong acid such as HCl, to stabilize 5-HIAA and catecholamines. VMA, HVA and total metanephrines stabilities are equivalent with HCl and citrate. On a practical viewpoint all the biogenic amines but EPI are stable in urines stabilized with citrate up to four weeks at 22 °C. We believe that this is not an issue since EPI is not secreted by neuroblastomas and because catecholamine measurement is not recommended for the diagnosis of pheochromocytomas. If EPI is required for another indication, it may be preserved in citrate for 48 h at 4 °C and four weeks at −20 °C. Moreover, using a buffer prevents excessive acidification of the urine for the first urination of a 24-hour collection. Indeed, the amount of stabilizer for 3 L of urine is usually placed in the collection bottle before urine collection. A large excess of preservative is therefore present after the first urine collection, which may result in a dramatic degradation with HCl, but not with citrate. A concentrated solution of citrate may also be used to stabilize urine spots from neonates for whom a suspicion of neuroblastoma requires the measurement of catecholamine, VMA, and HVA in a limited volume of urine. We have also shown that the addition of citrate does not result in analytical interference or bias when using LC-MS/MS methods.

Finally, sodium citrate is a non-harmful chemical easier to use by patients who are requested to collect a spot or 24-hour urines.

Of note, calibrators and quality controls (QC) from commercial furnishers have to be reconstituted in either water (e.g., Chromsystems' HPLC calibrators for VMA, HVA, 5-HIAA, and catecholamines, and QC samples for all nine metabolites) or in HCl (e.g., in HCl 0.2 M for the ClinRep kit for VMA, HVA, and 5-HIAA from RECIPE). The majority of external quality assessment (EQA) samples have to be reconstituted in water, such as the ones from the ProBioQual program, which pH is 6.5 after reconstitution, questioning the inter-laboratory accuracy proficiency testing for this pH -sensitive analyte.

In conclusion, we propose to replace HCl by sodium citrate for stabilizing urinary 5-HIAA, VMA, HVA, catecholamines and total metanephrines as a safe alternative for clinical chemistry laboratory and patients.

CRediT authorship contribution statement

Philippe J. Eugster: Conceptualization, Methodology, Formal analysis, Writing - original draft. **Catherine Centeno:** Investigation, Methodology, Writing - review & editing. **Marielle Dunand:** Investigation, Methodology, Writing - review & editing. **Caroline Seghezzi:** Investigation, Methodology, Writing - review & editing. **Eric Grouzmann:** Conceptualization, Writing - review & editing, Supervision.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cca.2020.12.009.

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