International Journal of KIU

Journal home page : https://www.kiu.lk/ijkiu/
DOI:10.37966/ijkiu2021021012

Original Article

# Effectiveness of currently used urinary preservatives in preserving high demand biochemical analytes. A study in the context of Sri Lankan laboratory setting

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Article history:
Received 23 March 2021
Received in revised form
25th May 2021
Accepted 27 June 2021

Cite as:

Hewapathirana P. L., Abeywardena H. N. G., Uluwaduge D. I., Amarasinghe N. S., Perera K. C., Bandara E. M. S. (2021). Effectiveness of currently used urinary preservatives in preserving high demand biochemical analytes. A study in the context of Sri Lankan laboratory setting. International Journal of KIU, 2 (1). 41-46. https://doi.org/10.37966/ijkiu2021021012

## Abstract

### Introduction

Analysis of 24-hour urine composition is widely used in diagnosis of acute and chronic kidney diseases. In Sri Lanka, no guidelines are available on preservation of urine. This study aimed to identify an effective preservation technique/s among currently practiced techniques in laboratories to maintain chemical stability of high demand bio chemical analytes in a 24-hour urine collection.

### Methods

An experimental study was undertaken using urine samples from volunteers (n=42, National Hospital of Sri Lanka and University of Sri Jayawardenepura, Nugegoda, Sri Lanka). Each sample was divided in to 10 ml aliquots; Out of the lot, one was preserved without any preservative and another refrigerated at 4ºC for 24 hours. Other aliquots were preserved for 24-hours by addition of following preservatives in a 24-hour collection: boric acid g/ urine (5, 7.5, 10), sodium azide g/ urine (0.3, 0.6, 0.9), HCl ml/ urine (1N;10ml, 6N;10ml, 25ml and 30ml). The aliquots with preservatives were kept at room temperature for 24 hours. Protein, Creatinine, $Ca^{2+}$, $Mg^{2+}$ and $PO_4^{3-}$ concentrations were measured in each aliquot. Least mean squared error for each analyte in different preservatives was calculated.

### Results

For protein and creatinine, least mean squared error was given by, 10g/L boric acid. That of $Ca^{2+}$, $Mg^{2+}$ and $PO_4^{3}$ were observed when the sample was refrigerated or after addition of 10g/L boric acid as the preservative.

### Conclusions

Sodium azide and HCl that are utilized in current practice as preservatives for 24-hour urine collections do not show better performance in selected analytes. Boric acid (10g/L) is more effective in persevering protein and creatinine. Due to practical issues in acquiring refrigeration facilities, $Ca^{2+}$, $Mg^{2+}$ and $PO_4^{3-}$ also can be preserved effectively by using the same preservative. Consequently, Boric acid (10g/L) could be recommended as an effective preservative to preserve selected analytes in 24-hour urine collection.

***Keywords:*** Urine preservatives, 24-hour urine collection, Boric acid, Creatinine.

## Introduction

Analysis of 24-hour urine has been widely used to focus on an appropriate basis for the decisions on the diagnosis of acute and chronic kidney diseases (Kumar & Clark, 2017). This is due to the fact that urine reveals a wide spectrum of diagnostically important biochemical information about the human body (Kumar & Clark, 2017; Burits & David, 2014). Therefore, accuracy and reliability of the routine laboratory investigations based on the methodological standardization of patient preparation, sample collection and preservation of 24-hour urine samples are important components of the preanalytical stage of the laboratory testing and reporting (Feres et al., 2011). In this context, accurate collection methodologies of any biological fluid are very important to avoid pre analytical errors and recurrent sample collections. Due to the prolong sample collection time, minimization of variations in urine composition is critically important to maintain the accuracy of laboratory investigations (Burits & David, 2014).

Protein, creatinine, pH, electrolytes, inorganic ions ( $Na^{+}$, $K^{+}$, $Cl^{-}$, $Ca^{2}$+, $Mg^{2+}$,$HCO^{3}$-, $PO_4^{3-}$, heavy metals (mercury, zinc, nickel), urea, amino acid, cortisol, cysteine, oxalate and uric acid are analytes that can be tested using 24 hour urine sample (Feres et al., 2011).

Bacterial action, chemical decomposition and atmospheric oxidation of unstable compounds are some problems encountered with periodical collection. Conversion of urea into ammonia by bacterial action gives unpleasant ammonical odour and causes difficulties in determination of urea, ammonia, pH and total nitrogen (Burits & David, 2014). Further bacteria utilize creatinine for their growth and convert it into creatine which may lead to underestimation of the test results (Burits & David, 2014).

The consistency of the biological sample is a major factor for optimal laboratory results. Therefore, various preservatives and preservation methods are used based on their physiochemical roperties in determination of biochemical analytes.

Refrigeration, use of HCl, boric acid, sodium azide, formaldehyde, thymol and toluene are commonly used preservation methods to inhibit degradation and to preserve urinary analytes (Thongboonkerd & Saetun, 2007). Refrigeration minimizes the rate of bacterial action and chemical decomposition (Burits & David, 2014). Boric acid has a bactericidal action against most urinary bacteria (Kumara et al., 2015; Meers & Chow, 1990). Though sodium azide is toxic to humans, it has a bacteriostatic effect by inhibiting cytochrome oxidase in gram negative bacteria (Walsh et al., 2003). Thymol also has an antibacterial activity because of it's phenolic structure (Iqbal et al., 2015).

Selection of an appropriate preservative depends on the analyte that needs to be investigated. Although different strategies have been reported in the literature, the effectiveness in preservation of biological analytes is questionable. Feres et al., (2011) conducted a clinical trial using 24-hour urine samples of 22 volunteers, which were collected in HCl as the preservative under three conditions. According to the results, it has been concluded that protein did not show accurate values in the presence of acid preservatives and analytes which require acid preservatives (creatinine) showed acceptable values in acidification. Ferraz et al., (2006) carried out a study on 34 healthy subjects and preserved 24-hour consecutive duplicate urine samples (pre delivery acidified samples and post-delivery acidified samples) with 6 mol HCl to analyze $Ca^{2+}$, oxalates, uric acid and creatinine. Same procedure was carried out with spot urine samples with HCl and $NaHCO_3^{-}$. The results revealed that there was no significant difference between pre and post-delivery acidification results. This made the conclusion that acidification is not needed for the preservation of above mentioned analytes.

Another study was conducted by Sodi et al., (2009) to identify the necessity of acidification of urine before measuring $Ca^{2+}$. The study recruited 133 patients to collect paired 24-hour urine samples. Samples were preserved with 5 mol/L HCl and the $Ca^{2+}$ levels obtained were compared with that of unpreserved urine in the same pair. The results suggested that acidification was not necessary at pre analytical stage to measure $Ca^{2+}$.

A study was carried out to evaluate the chlorhexidine/n - propyl gallate as a urine preservative using pooled urine to detect long term storage ability (Nillen & Smith, 2004). It was found that the mixture had the ability to store urine without changing several parameters including $Ca^{2+}$ and creatinine.

Yilmaz et al., (2008) investigated the necessity of a preservative in collection of 24-hour urine samples. Twenty-four hour collections (n=50) and spot urine samples (n=20) were preserved with HCl and $NaHCO_3^-$. Results showed that addition of a preservative was not necessary at pre analytical stage to measure $Ca^{2+}$, $Mg^{2+}$, $PO_4^{3-}$ and uric acid. A case study reported that accidental ingestion of sodium azide in 24-hour urine container resulted in cardio vascular collapse and death (Herbold et al., 1995). Therefore, it was concluded that refrigeration is more suitable to avoid accidental ingestion of harmful preservatives. Bacteriostatic effect of boric acid was evaluated in another investigation and the results showed that 10g/L boric acid is weakly bactericidal and 10 -20 g/L boric acid is more effective (Meers & Chow, 1990). In this context the studies which have been conducted until now, don't focus on providing any recommendation on superior methods to preserve urine for investigation purpose of biological analytes.

However, due to low temperatures and favorable climate in western countries, urine samples are stored at room temperature in which the analytical procedures are carried out. Even though storing urine samples at room temperature is recommended for some analytes in international guidelines, it is difficult to adopt in Sri Lanka due to temperature variations that favours the bacterial growth. When urine samples are stored at room temperature, the abnormal ammonical (pungent) odour comes out from older urine samples due to conversion of urea into ammonia by the action of bacteria (Burits & David, 2014). It may cause difficulties in carrying out analytical procedures. In turn, refrigeration could not always affordable In turn, poor communities in Sri Lanka cannot afford refrigeration despite its wider applicability in other countries.

Measurement of protein and creatinine concentrations in 24-hour urine collection is predominant in urinalysis. Nevertheless, in Sri Lankan laboratories, several protocols for urine analysis have already included vast variety of preservatives to prevent physiochemical and biological changes during urine collection (Table 1) whereas some others have not. Unavailability of a single preferred method to preserve biochemical analytes in a 24-hour urine sample is a major drawback in this line of investigations. Therefore, at present the patient is required to collect multiple urine samples for different analytes. Also, a scientific study has not been conducted in Sri Lanka to support or to make any recommendations on methods to be adopted to preserve a 24-hour urine sample. In this context, standardizations for urine collection and appropriate sample preparation methods are, therefore, crucially required.

Thus, this study aimed to identify an effective preservation technique/s among currently practiced techniques in chemical pathology laboratories to maintain chemical stability of high demand bio chemical analytes (e.g. protein, creatinine, calcium, magnesium and phosphorus) in a 24-hour urine collection. Further an attempt was taken to acclaim an appropriate method/s to minimize patient discomfort in collection of multiple samples for different analytes. Preservatives for the study were selected from the currently available list used in chemical pathology laboratories of Sri Lanka.

Table 1: In-use preservatives for 24-hour urine collection in Healthcare Institutions, Sri Lanka

| Analyte to be preserved | Preservative |
|---|---|
| Protein | 98% HCl<br>5g Boric acid<br>10g Boric acid<br>10% Thymol (2 ml)<br>10 g Thymol<br>Few crystals of Thymol<br>10% Thymol (1 ml)<br>10% Thymol (10 ml) in isopropanol<br>0.5 g Sodium azide<br>Toluene 30 ml |
| Creatinine | 6 mol/HCl (10 ml)<br>6 mol/HCl (25 ml)<br>98% HCl (10 ml)<br>Concentrated HCl (10 ml)<br>6 N HCl 30 ml<br>10% HCl 10 ml<br>20 % Acetic acid (20 ml)<br>10g Boric acid<br>5g Boric acid<br>10% Thymol (2ml) in isopropanol<br>10% Thymol (10ml) in isopropanol<br>Toluene (30 ml) |
| $Ca^{2+}$, $Mg^{2+}$, $PO_4^{3-}$ | 6 mol/HCl 10 ml,<br>6 mol/HCl 25 ml<br>98% HCl 10 ml<br>Concentrated HCl 10 ml<br>6 N HCl 30 ml<br>10g Boric acid<br>20 % Acetic acid (20 ml)<br>Toluene (30 ml)<br>Few crystals of sodium azide<br>Toluene 30 ml<br>10% Thymol 10ml in isopropanol<br>Few crystals of sodium azide |

## Materials and Methods

### Subjects

The study was conducted at the chemical pathology laboratory of the National Hospital of Sri Lanka. Forty-two (42) participants were enrolled after obtaining the written consent. The study was ethically approved by the Ethics Review Committee, Faculty of Medical Sciences, University of Sri Jayewardenepura (Protocol approval No. MLS 11/2015) and Ethics Review Committee, National Hospital of Sri Lanka. Patients who were already diagnosed with acute or chronic kidney diseases in renal unit and nephrology clinic of National Hospital of Sri Lanka and age-related healthy volunteers from university population were included in the study. Study population was selected to represent normal and high values of urinary analyte concentrations anticipated to measure.

### Methods

Each sample was divided into 12 aliquots, each containing 10 ml urine as detailed in the Figure 1. First sample was tested immediately without preservatives for protein, $Ca^{2+}$, $Mg^{2+}$ and $PO_4^{3-}$. Second sample without any preservative was stored at $4^0$C (refrigerator) for 24 hours. The other 10 aliquots were preserved for 24 hours at room temperature after addition of preservatives. Preservatives were selected based on currently in-use preservatives of chemical pathology laboratories of Sri Lanka (Table 1). Samples were analyzed for protein, creatinine, $Ca^{2+}$, $Mg^{2+}$, $PO_4^{3-}$ post preservation of 24 hours. A detailed schematic representation of the methodology is depicted in Figure 1.

The results were analyzed by using SPSS to derive mean squared error of each analyte in different preservatives in comparison with that of unpreserved sample (freshly voided urine sample). The preservation method which had the least mean squared error was considered as the best preservation method (among the other tested methods) for the corresponding urinary analyte.

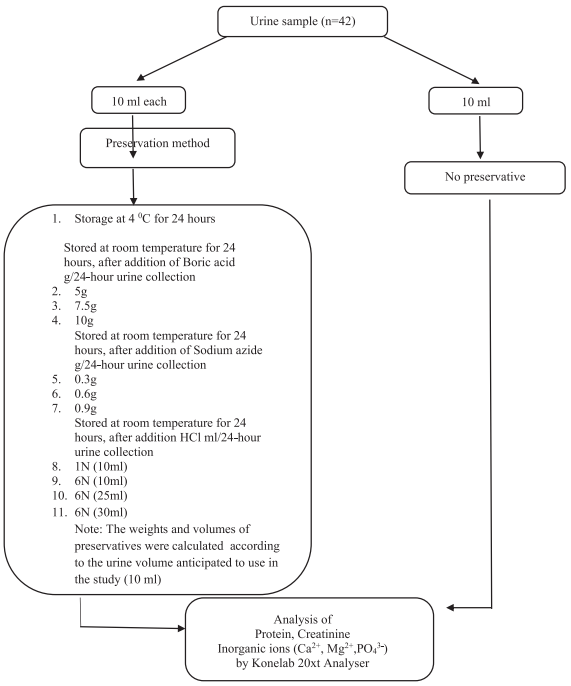


Figure 1: Schematic summary of the study design adopted in the present study

## Results and Discussion

Urine may be a waste product, but it contains an enormous amount of information. Well-standardized procedures for collection, transport, sample preparation and analysis should become the basis of an effective diagnostic strategy for urinalysis. As reproducibility of urinalysis has been greatly improved due to recent technological progress, preanalytical requirements of urinalysis have gained importance and have become stricter. Since the patients themselves often sample urine specimens, urinalysis is very susceptible to preanalytical issues. Various sampling methods and inappropriate specimen transport can cause important preanalytical errors. The use of preservatives may be helpful for particular analytes. Unfortunately, a universal preservative that allows a complete urinalysis does not exist yet. In Sri Lanka different preservatives are used to preserve 24-hour urine collections as shown in table 1. According to the current practices adopted in Sri Lanka, analysis of high demand biochemical analytes requires collection of multiple samples which is inconvenient to the patients. The aim thereby was to select an ideal preservative which can be used to preserve most analytes to avoid discomfort to the patient.

According to the present study, the least mean squared error for protein and creatinine was permitted when urine was preserved with 10 g boric acid / 24-hour urine collection (Table 2). Therefore, among other methods, addition of boric acid (10 g) was moderately effective in preserving proteins and creatinine, though this method was not used in most of the Sri Lankan government institutions. Addition of HCl as the preservative for urinary protein showed the highest mean squared error. However, it was the method that is in practice at some institutions.

The findings of the present study are supported by a similar study conducted by Feres et al., (2011) concluding that analysis of microalbumin and protein in 24-hour urine samples did not show good performance in the presence of acid preservatives.

The present findings are inconsistent with the study carried out by Ferraz et al., (2006) indicating that acidification is not necessary for creatinine assay. Further, the present study revealed that the refrigeration did not show better performance for analysis of protein and creatinine in 24-hour urine samples.

The study suggests that, for the analysis of inorganic ions, the most effective preservation method was refrigeration at $4^0$C due to the lowest mean squared error observed (Table 2). A similar study has been carried out by Yilmaz et al., (2008) and supports the findings of the present analysis indicating that no preservatives are needed for the assay of inorganic ions in 24-hour urine samples. The next lowest mean squared error was obtained by 10g boric acid / 24-hour collection for inorganic ions (Table 2). The same preservative showed the best preservation performance with protein and creatinine in the present study. In some communities of Sri Lanka refrigeration facilities are not available due to economic hindrances. Therefore, 10g boric acid / 24 hour collection may have the potential to preserve urinary inorganic ions also, effectively. Since sodium azide has toxic effects and also proven to have a higher mean squared error, the present study does not recommend sodium azide as a preservative in 24-hour urine collections, though it is currently used in different institutions in Sri Lanka (Herbold et al., 1995).). To the best of our knowledge, this data set is the first that provides the direct evidence of effectiveness of chemical stability on the urinary analyte profile. Our present study has addressed an important issue of “sample collection and storage” prior to the analysis of high demand biochemical analytes in urine.

Table 2: Mean squared errors of each measured analyte in urine when preserved in different preservatives (in comparison with analytes in freshly voided urine)

| Preservative / Analyte | Storage at 4°C | 1N HCl 10ml | 6N HCl 10ml | 6N HCl 25ml | 6N HCl 30ml | Boric Acid 5g | Boric Acid 7.5g | Boric Acid 10g | Sodium Azide 0.3g | Sodium Azide 0.6g | Sodium Azide 0.9g |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Protein | 71.4888 | 78.3831 | 73.6648 | 58.6461 | 73.6499 | 32.4121 | 48.9231 | 31.3597* | 76.2053 | 42.6796 | 71.0604 |
| Creatinine | 0.1071 | 0.0857 | 0.0760 | 0.0779 | 0.4448 | 0.2083 | 0.0955 | 0.0662 * | 0.1679 | 0.0895 | 0.3179 |
| $Ca^{2+}$ | 0.0045 ** | 0.0096 | 0.0060 | 0.0079 | 0.0272 | 0.0173 | 0.0056 | 0.0050 *** | 0.0072 | 0.0251 | 0.2244 |
| $Mg^{2+}$ | 0.0169 ** | 0.0279 | 0.0237 | 0.0196 | 0.0190 | 0.0218 | 0.0284 | 0.0194 *** | 0.0251 | 0.0236 | 0.0274 |
| $PO_4^{3-}$ | 0.1812 ** | 0.3358 | 1.9729 | 0.7964 | 0.2988 | 0.4583 | 0.3248 | 0.2481 *** | 0.4490 | 0.7455 | 0.5831 |

*Least mean squared error for protein and creatinine; Boric acid 10g

**Least mean squared error for $Ca^{2+}$, $Mg^{2+}$ and $PO_4^{3-}$; Refrigeration

***Least mean squared error next to refrigeration for $Ca^{2+}$, $Mg^{2+}$ and $PO_4^{3-}$ ; Boric acid 10g

## Conclusion

On the basis of the data reported herein, our recommendation is to preserve protein and creatinine by addition of boric acid 10g /1L, 24 hour urine collection. Even though, other inorganic ions ($Ca^{2+}$, $Mg^{2+}$ and $PO_4^{3-}$) are more preserved in refrigerated samples, boric acid 10 g/L in 24-hour urine collection may be used as most suitable preservative with better preservation ability of all the analytes in a urine sample. Further, this can be implemented even in a peripheral setup where there are no refrigeration facilities. When a single preservative is used to preserve multiple analytes that have been ordered by the clinician, the patient needs to collect a 24-hour urine sample only once. This will omit the necessity of collection of several 24-hour collections with different preservatives as in current practice. This will minimize the discomfort which afflicts patient and is more important in critical care patients.

## Conflicts of interest

There is no conflict of interest.

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