Lithostatine concentration is not useful for assessing the preanalytical variations in biobanked urine samples.

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Abstract

Proteomic research requires high quality and standardized samples. Quality control (QC) biomarkers, which are sensitive to the collection, processing or storage conditions, would be useful tools to identify compromised samples. This study evaluates the usefulness of renal lithostatine as a QC tool for urine sample processing in daily biobank work. Four factors (pre-analytical variations) were examined for their effects on renal lithostatine as measured by ELISA: time from sample collection to centrifugation, number of specimen freeze-thaw cycles, specimen preservation with protease inhibitors and the inclusion or exclusion of urinary sediment.

Introduction

Urine proteins are suitable indicators of renal function and human health in general. Proteomics involves the systematic analysis of proteins, peptides, their modifications, quantities and functions in order to better understand disease prognosis and diagnosis. Urine is a complex source of candidate protein prognostic and diagnostic biomarkers associated with diseases, e.g. cancer [1, 2]. Proteomic analysis involves protein extraction, separation, identification and quantification [1]. However, analysis of the urine proteome is affected by high intra- and inter-individual variability of urine specimens in terms of presence and concentration of numerous low abundance proteins or modified protein forms.

Urine handling and storage can also affect proteomic analysis results. Freeze-thaw cycles can significantly alter the profile of detected proteins, particularly those in the lower molecular weight fraction [3, 4]. Prolonged storage at ambient temperatures also increases the variation in the protein profile [5]. Therefore, proteomic research requires high quality and standardized urine samples. The knowledge of the pre-analytical variations is necessary for further comparative proteomic research in order to compare urine samples that are compatible from a pre-analytical point of view. Quality control (QC) biomarkers, which are sensitive to the collection, processing or storage conditions, are useful tools to identify compromised samples. Ideal QC biomarkers should be ubiquitous and showing an on/off response to a specific preanalytical variation [6]. Lithostatine (or Lithostathine-1-alpha, or Regenerating protein 1 α, or REG1α), a low abundance protein in urine, has been suggested as a possible sensitive QC biomarker (QC tool) for urine sample processing [7].

Lithostatine is a secretory protein, synthesized by pancreatic acinar cells, first described as a constituent of pancreatic stones, and controls $CaCO_3$ crystal growth. It was also reported that, in the thin descending limb of the Henle's loop, urine was supersaturated in $CaCO_3$ [8]. This observation suggested the presence in the kidney of a similar inhibitor of calcium carbonate precipitation and $CaCO_3$ crystal growth. Verdier J. M. et. al. [9] showed that a protein immunologically related to lithostatine is actually present in urine of healthy subjects and in renal stones. Because of its structural and functional similarities with pancreatic lithostathine, it was called renal lithostatine.

Our current study focused on renal lithostatine, in order to validate its usefulness as a quality control tool for urine sample processing in daily biobank work. Four factors (pre-analytical variations) were examined for their effects on renal lithostatine level as measured by ELISA: time from sample collection to centrifugation, number of specimen freeze-thaw cycles, specimen preservation with protease inhibitors and the inclusion or exclusion of urinary sediment.

Materials and Methods

Urine samples were collected from 6 healthy volunteers who had signed an informed consent form for biospecimen research purposes. The study design was the following:

- First phase:
  urine samples from 3 healthy donors (two females, one male) were examined for two pre-analytical factors - time from sample collection to centrifugation, number of specimen freeze-thaw cycles,
- Second phase:
  urine samples from 3 healthy donors (two females, one male) were examined for four pre-analytical factors - time from sample collection to centrifugation, number of specimen freeze-thaw cycles, specimen preservation with protease inhibitors and the inclusion or exclusion of urinary sediment.

Midstream urine samples were collected in a dry sterile polypropylene urine container, without protease inhibitors in order to make eventual degradation of lithostatine easier to assess, and with protease inhibitors (Roche Diagnostic, ref. 05056489001) in the second phase of the study. Between collection and first centrifugation, the urine samples stood at 4°C. Pre-centrifugation delay was 2h, 4h, 24h or 48h for each urine sample. Additionally, in the second phase of the study, each urine sample was divided in two parts: one underwent centrifugation, the other not. Urine aliquots were placed in a previously refrigerated centrifuge and spun at 2 000 g for 10 minutes (at 4°C) to separate urine supernatant from cells and cell debris. The supernatants were transferred to new polypropylene tubes and centrifuged again at 12 000 g for 10 minutes (at 4°C) to eliminate further cell debris and microparticles. Aliquots of the second centrifugation supernatant and corresponding urine samples without centrifugation were transferred to -80°C in polypropylene cryovials, and subjected to one, five or ten freezing-thawing cycles. All samples were tested by REG 1α

ELISA (AMSBIO, AMS.E01R0033) according to the manufacturer's instructions. Briefly, those wells that contain REG 1α and enzyme-conjugated antibody exhibited a change in colour. The enzyme-substrate reaction was terminated by addition of a sulphuric acid solution and the colour change was measured spectrophotometrically at a wavelength of 450 nm. A standard curve was plotted relating the intensity of the color (OD_{450}) to the concentration of standards included in the assay. The REG1α concentration in each sample, measured in duplicate, was calculated from this standard curve. Data are presented as the mean ± SD. Statistical analyses were performed by ANOVA analysis using SigmaPlot 12.0 software (Systat Software Inc, USA) and $P < 0.05$ was considered statistically significant.

Results and Discussion

Current best practices describe urine sample collection and processing and propose urine QC markers/assays [7]. In our study we have investigated renal lithostatine as a previously reported potential QC marker for urine sample processing. We first studied the impact of the time delay between sample collection and centrifugation and of sample freeze-thaw cycles on renal lithostatine concentrations (Fig.1).

We found no statistical difference due to the number of freeze-thaw cycles. Similarly, there was no statistically significant difference in lithostatine levels between different pre-centrifugation delays, up to 48 hours (Fig.1). Although functional proteases are unlikely to be found in urine, many biobanks use protease inhibitors when collecting urine specimen. Therefore, we also assessed the potential impact of the use of protease inhibitors (PIs). It has also been reported [7] that lithostatine cannot be detected in the absence of protease inhibitors, and that detection of this protein is hampered by multiple freeze-thaw cycles. Therefore, in the second phase of the study we also evaluated the levels of lithostatine in urine samples collected with protease inhibitors. Another factor that we have tested was the inclusion or exlusion of urinary sediment. Urinary sediment may contain different types of cells [10], and cell-derived proteins, which may interfere with the lithostatine immunoassay.

In the second phase of the study, addition of protease inhibitors showed no significant effect on lithostatine levels, when urine was subjected to the same pre-analytical conditions: pre-centrifugation delays and freeze-thaw cycles (Table 1).

Urinary sediments were generally found to have slightly higher lithostatine concentrations than the supernatants, although this effect was not statistically significant (Table 1). The urine sediment may contain a source of lithostatine or may interfere with the immunoassay. It is indeed recommended to centrifuge and remove the sediment from the urine samples before proteomic investigations and/or storage [7, 11].

The second phase of the study confirmed that neither pre-centrifugation delays up to 48 hours nor multiple freeze-thaw cycles had a significant impact on lithostatine levels.

In our study, the level of lithostatine in urine samples, processed under different pre-analytical conditions, was measured. The results showed that the level of lithostatine did not change significantly in any of the applied conditions. Therefore, lithostatine concentration,

when measured by ELISA, cannot indicate alterations linked to processing time and storage conditions and cannot be used as a quality control tool (marker) for assessment of the integrity of urine samples. The capture antibodies used in this method were monoclonal antibodies, with affinity to all lithostatine isoforms, as confirmed by the supplier. However, low specificity for certain lithostatine isoforms found in urine, might explain the very low concentrations measured in the study.

Our results support previous observations that several biomarkers can remain stable in urine over long periods of time [12]. In conclusion, our study suggests there is an urgent need to define an appropriate urine quality control tool, in order to enable validation of retrospective urine collections and standardisation of urine sample processing in biobanks.

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Figure 1

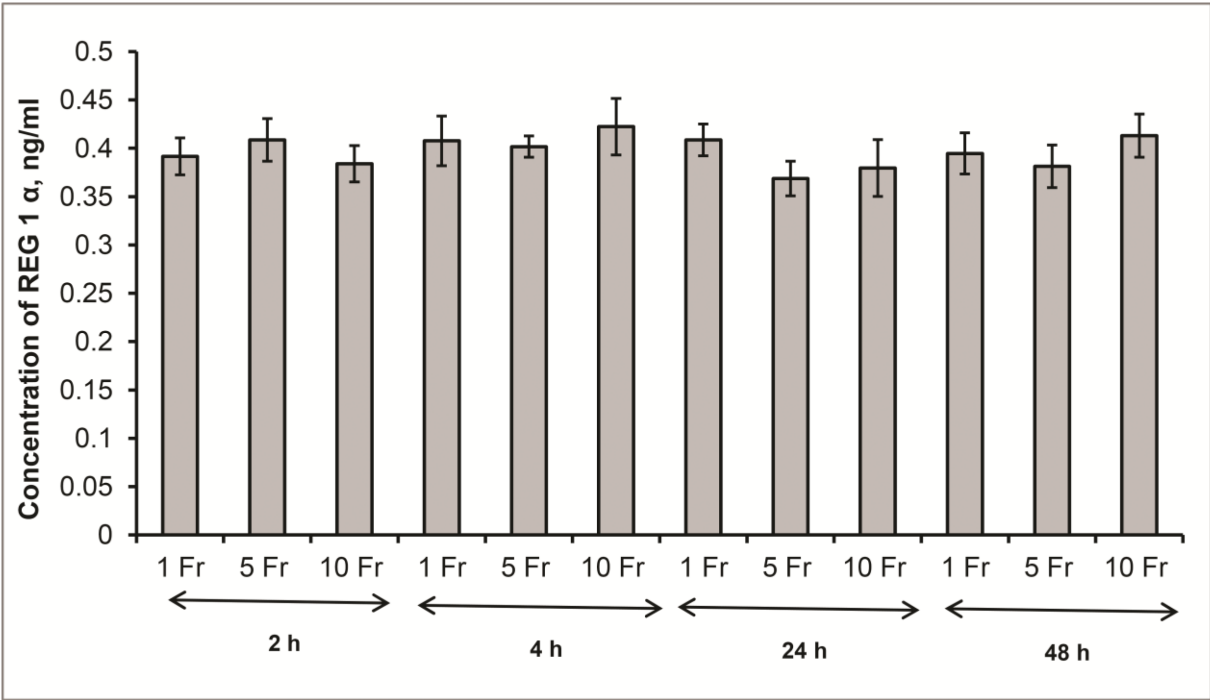

Table 1

Precentrifugation delay, hours	1 freeze-thaw cycle				5 freeze-thaw cycles				10 freeze-thaw cycles			
	with centrifugation		without centrifugation		with centrifugation		without centrifugation		with centrifugation		without centrifugation	
	with PIs	without PIs	with PIs	without PIs	with PIs	without PIs	with PIs	without PIs	with PIs	without PIs	with PIs	without PIs
0	0.170 ± 0.037	0.163 ± 0.037	0.157 ± 0.010	0.177 ± 0.012	0.126 ± 0.021	0.129 ± 0.022	0.141 ± 0.025	0.136 ± 0.028	0.117 ± 0.014	0.119 ± 0.002	0.126 ± 0.018	0.133 ± 0.026
4	0.147 ± 0.032	0.143 ± 0.020	0.163 ± 0.033	0.161 ± 0.038	0.160 ± 0.034	0.150 ± 0.047	0.170 ± 0.051	0.177 ± 0.025	0.128 ± 0.019	0.128 ± 0.027	0.146 ± 0.017	0.140 ± 0.029
24	0.195 ± 0.027	0.223 ± 0.049	0.200 ± 0.060	0.172 ± 0.038	0.156 ± 0.013	0.139 ± 0.009	0.173 ± 0.032	0.174 ± 0.016	0.174 ± 0.034	0.160 ± 0.052	0.102 ± 0.011	0.187 ± 0.054
48	0.122 ± 0.011	0.131 ± 0.030	0.158 ± 0.044	0.160 ± 0.041	0.140 ± 0.020	0.141 ± 0.050	0.150 ± 0.044	0.154 ± 0.040	0.148 ± 0.021	0.137 ± 0.034	0.153 ± 0.028	0.157 ± 0.031