Spectrophotometric Method for the Determination of Total Thiols in Human Urine

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Abstract. Thiols have been of enduring interest for many years because of their role in biological and pharmacological processes. Monitoring of total thiols content is very important in order to understand their function in living organisms. This paper describes the spectrophotometric method for the determination of total thiols concentration in urine. The method is based on derivatization with 1-benzyl-2-chloropyridinium bromide and ultraviolet detection of S-pyridinium derivatives at 316 nm. The analytical recovery and RSD values for precision within the calibration range were from 95.7 to 102.9% and from 2.1 to 8.4%, respectively. The concentration of total thiols normalized against creatinine for 38 apparently healthy subjects (19 women and 19 men) occurred in the range 17.2–73.7 and 25.7–83.6 mmol/mol creatinine, respectively. There was no difference in the urinary excretion of thiols in men and women, but there was a significant statistical correlation between urine total thiols and age in the studied group.

Key words: estimation, cysteine, cysteinylglycine, homocysteine, UV spectroscopy.

Introduction

Thiols are products of sulfur metabolism that play an important role in the metabolic processes of all living organisms [1]. Thiol and disulfide drugs are potential chelating agents for mercury poisoning, but endogenous thiols, such as cysteine, homocysteine, and glutathione play an important role in the distribution of mercury throughout the body [2]. Some critical reviews have demonstrated that aminothiols are involved in the pathogenesis of human diseases [3]. When cellular processes are affected, changes in the amount of thiols and disulfides in body fluids have been observed. Therefore, analytical monitoring of thiols and disulfides is crucial in order to understand their physiological and pathological functions.

There are some spectrophotometric methods for the determination of thiols in pharmaceutical products [4-7], but these methods are not applicable to other areas of research. These methods are based on the different reactions of thiols with several reagents, e.g. eosin-silver(I)-adenine ternary complex [4], copper(II) in the presence of 2,9-dimethyl-1,10-phenanthroline [5], ammonium molybdate in the presence of sulfuric acid [6], N,N-dimethyl-p-phenylenediamine in acidic solution, in the presence of iron(III) ions [7]. Some spectrophotometric methods allow the determination of reduced and total thiols in biological samples, such as rat brain [8], plasma [9], and urine [10]. These methods use reactions of thiols with gold nanoparticles [8,10] or copper(II)-xylanol orange complex [9]. Moreover spectrophotometric methods can be used to determine simple thiols and protein sulphydryls by reaction with esters of propiolic acid [11] or Ellman’s reagent (DTNB) [12]. Ellman’s spectrophotometric method [13] still remains the most popular thiol assay method [12,14]. DTNB is itself a disulfide compound that interferes with the reduction agent (e.g. dithiotreitol, mercaptoetanol, and phosphines) and thus does not provide an accurate result for the amount of thiols in the body. Therefore, each procedure for determination of total thiols based on DTNB must include a step for removal of excess reducing agent.

1-Benzyl-2-chloropyridinium bromide (BCPB) has previously been used as the derivatization reagent for determination of aminothiols and thiol drugs by HPLC. Methods using BCPB are based on the conversion of the thiols to highly UV-absorbing derivatives by a reaction with the reagent, which is followed by the separation and quantiation by an ion-paired reversed-phase HPLC [15-18].
The goal of this study is to develop a spectrophotometric method for the determination of total thiols in human urine. The method relies on a reaction of thiols with the thiol-specific tagging reagent, BCPB, to form stable UV-absorbing derivatives.

Experimental Procedure

Chemicals. 1-Benzyl-2-chloropyridinium bromide (BCPB) was synthesized in our laboratory as described earlier [15]. L-cysteine (CSH) and DL-cystine (CSSC) were obtained from Reanal (Budapest, Hungary). Hydrochloric acid, perchloric acid (PCA), sodium hydroxide, and tris(hydroxymethyl)aminomethane (TRIS) were from J.T. Baker (Deventer, Netherlands). Creatinine, cysteinylglycine (CGSH), DL-homocysteine (HCSH), DL-homocystine (HCSCH), picric acid, and tris(2-carboxyethyl)phosphine hydrochloride (TCEP) were from Sigma (St. Louis, USA). Trichloroacetic acid (TCA) was from Merck (Darmstadt, Germany). Stock standard solutions were prepared by dissolving an appropriate amount of thiols and their symmetrical disulfides (final concentration 0.1 mol/L) in 0.02 mol/L HCl, BCPB (0.1 mol/L) in water, and TCEP (0.25 mol/L) in TRIS buffer (pH 9). Stock solutions of thiols, disulfides, and BCPB were kept at 4 °C for several days without noticeable change of content. TCEP solution and mixture of 0.8% picric acid with 6.5% NaOH (1:1, v:v) were prepared daily before analysis. The working solutions were prepared by dilution with water as needed.

Apparatus. All absorption spectra were made using Hewlett-Packard HP 8453 (Waldbronn, Germany) diode array UV-Vis spectrophotometer with a slit width of 1 nm, equipped with 10 mm matched quartz cells. Water was purified using a Millipore Milli-QRG system (Vienna, Austria). All experiments were performed at 25 °C.

Sample collection. The first morning urine samples were donated by 38 apparently healthy, ethnically homogeneous volunteers after overnight fasting. During the study, no concomitant medications were allowed. Informed consent was obtained from volunteers, and this study was approved by the Bioethics Committee of the University of Lodz. Urine samples were processed the same day or stored at -20 °C until analysis. On the day of analysis, samples were thawed and processed as described below.

Recommended procedure for total thiols determination. For the measurement of total thiols, 250 μL of urine was mixed with 100 μL of pH 9 (0.2 mol/L) TRIS buffer, 10 μL of 0.25 mol/L TCEP. After a 15 min reduction at room temperature, 20 μL of 0.1 mol/L BCPB was added, vortex-mixed, and kept at room temperature for 10 min. The mixture was acidified with 50 μL of 3 mol/L PCA and made up to a final volume of 2 mL with water. The solution was transferred to a quartz cell and the absorption was measured at 316 nm against urine blank solution (without BCPB).

Calibration. To prepare the calibration standards used to determine total thiols in human urine, portions of 250 μL urine were placed in a sample tube and spiked with the growing amount of the working standard solution of cystine to provide, assuming 100% of the future reduction of the disulfide bonds, concentration of exogenous cysteine of 0, 50, 100, 200, 300, 400, and 500 μmol/L urine. The calibration standards were processed in triplicate according to the recommended analytical procedure. The calibration curve was obtained by least-squares linear regression analysis of the cysteine derivative absorbance versus concentration. The slope of the calibration curve was used to calculate the thiol concentration in urine samples.
Creatinine determination. The urinary creatinine was determined by a spectrophotometric method based on the alkaline picrate of Jaffé reaction [19]. For analysis of creatinine, 100 μL of urine was diluted 1:50, after which 500 μL of solution was mixed with 500 μL of 20% TCA and 1000 μL mixture of 0.8% picric acid and 6.5% NaOH (1:1, v:v). After 20 min, the solution was transferred to a quartz cell and the absorption was measured at 520 nm.

Results and Discussion

The development of direct spectrophotometric methods for determination of thiols is still a difficult task. The problem in developing a suitable method is finding a reaction with a simple procedure that yields minimal side-products and interferences. Urine total thiols exist in different forms that consist of both oxidized (symmetric and mixed disulfides) and reduced fractions. To determine the total concentration of thiols, it is necessary to cleave disulfide bonds in order to obtain the free -SH group. For this purpose, TCEP was used in this study. As described previously, in standard water solution TCEP reduced disulfide bonds within 5 min at pH 9 and room temperature with 10-fold TCEP excess [20]. For urine samples, the reduction time was extended to 15 min.

Derivatization of thiols. Since thiols lack strong chromophores, the analytical methods require derivatization for their spectrophotometric determinations. Thiols react with BCPB (Figure 1) in a water environment to form 2-S-pyridinium derivatives, in the stoichiometric ratio of 1:1. The reaction is accompanied by an analytically advantageous bathochromic shift of the absorption maximum from 274 nm for the reagent to 316 nm for the derivative. The derivatization reagent itself does not absorb in this ultraviolet region [17]. The derivatization reaction yield was optimized in terms of pH, reagent excess and time. The results show that the optimal reaction pH for derivatization of thiols with BCPB occurred in the range 8.0-11.0 (Figure 2), and the reaction was complete after 5 min with 10-fold reagent excess at room temperature. For routine derivatization, pH 9 and time 10 min were chosen as optimal conditions for determination of thiols in urine samples.

Method validation. The method validation procedure encompassed linearity, precision, recovery, limit of detection, and quantitation. The main urinary thiols are cysteine, cysteinylglycine, and homocysteine [21]. The equations for the linear regression curves for cysteine, cysteinylglycine, and homocysteine, in the range $10^{-5} - 2 \times 10^{-4}$ mol/L, were $y = 8202c + 0.0068$, $y = 7902c - 0.0114$, and $y = 8014c - 0.0007$, respectively. The experiment has shown that the molar extinction coefficient for cysteine, cysteinylglycine, and homocysteine derivatives was around 8,000 dm$^3$/cm•mol. Because cysteine and cystine are the main thiol and disulfide excreted in the urine [21], cystine standard was chosen as a calibrator for preparation of the calibration curve for total thiols determination. Cystine concentration was recounted for cysteine, assuming complete reduction of the disulfide bonds.

![Table 1. Evaluation of the intra-day and inter-day precision, recovery and accuracy for thiols in PBS and human urine obtained by the proposed method, n = 3.](image-url)
The relationship between absorbance and cysteine concentration was continuous and reproducible, as demonstrated with a seven-point calibration curve. At each concentration, three replicates of urine spiked with cystine were tested. The calibration curve was linear in the range tested from 50 to 500 μmol/L. The equation for the linear regression line was y = 6.27E-4c + 2.53E-1; outliers were not excluded. The coefficient of correlation for the calibration regression was 0.9986.

In order to judge the quality of the elaborated method, precision and recovery were determined. The intra- and inter-day precisions and recoveries were measured in urine and phosphate buffered saline (PBS) samples spiked with cystine in three replicates. The inter-day precisions and recoveries were evaluated on three consecutive days in a week. As shown in Table 1, with no outliers excluded, the intra-day and inter-day imprecisions (RSD) were within 1.4-3.5% and 1.0-3.8% for the PBS samples, and within 2.1-8.6% and 3.4-8.4% for the urine samples, respectively. The recovery values were from 95.4% to 102.9%. This indicates that the proposed method gives accurate results.

The limit of detection (LOD) of an analytical procedure is the lowest amount of an analyte in a sample that can be detected, but not necessarily quantitated. The limit of quantitation (LOQ) is the lowest concentration of analyte in a sample that can be determined with acceptable accuracy and precision under the stated operational conditions of the method. LOD and LOQ of the presented method were established to be 14.5 and 48.4 μmol/L thiols in urine, respectively. LOD and LOQ were calculated according to the following equations [22]:

\[
\text{LOD} = 3 \frac{S_a}{b} \\
\text{LOQ} = 10 \frac{S_a}{b}
\]

Where, \(S_a\): the standard deviation of the intercept of regression line; \(b\): slope of the calibration curve.

**Application of the method.** The proposed method was applied to the determination of urinary excretion of total thiols in humans. Urine was received from 38 volunteers (19 women and 19 men), 14-82 years old. Analytical results are shown in Table 2. To facilitate comparison for different individuals, the analytical results for the urinary total thiols were normalized against creatinine. Concentrations of total thiols in human urine from 38 subjects varied from 17.2 to 83.6 mmol/mol creatinine with an average of 46.4 ± 14.4 mmol/mol creatinine. The obtained results are in good agreement with those reported by authors who have used the HPLC method [21,23,24]. Figure 3 is a plot of the individual values of urine total thiols normalized against creatinine versus age. There was a significant statistical correlation between urine total thiols and age in the studied group (r = 0.661, p < 0.05). Our previous data has shown that there were no significant differences between age and concentration of cysteine and cysteinylglycine excreted in urine [21]. Pastore et al [24] observed that homocysteine and cysteine did not change substantially throughout the different age groups in males, but increased in young females. The proposed method allows for the determination of the sum of all compounds possessing the -SH function excreted in urine, not only cysteine, cysteinylglycine, and homocysteine. The most current work of other authors [23,24], has shown that there is no difference in the urinary excretion of thiols in men and women, which agrees with the current results (p > 0.05).
Table 2. Total thiol concentration in urine from 38 volunteers.

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<tr>
<th></th>
<th>Female (n = 19)</th>
<th>Male (n = 19)</th>
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<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Range</td>
</tr>
<tr>
<td>Age (year)</td>
<td>42.3 ± 20.6</td>
<td>15 – 82</td>
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<tr>
<td>Thiol (μmol/L)</td>
<td>589.0 ± 165.4</td>
<td>284.5 – 810.1</td>
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<tr>
<td>Thiol/Creatinine (mmol/mol)</td>
<td>45.6 ± 13.7</td>
<td>17.2 – 73.7</td>
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</table>

Conclusion

A simple, sensitive, and rapid spectrophotometric method for determination of biologically active thiols in urine was established. This procedure is based on the derivatization of thiols with BCPB. The method can be used for routine clinical monitoring of total thiols in urine. The implementation of this method is facilitated because it uses a UV-Vis spectrophotometer, which is standard instrumentation in hospital laboratories and known for its stability and low demands in terms of maintenance.

Acknowledgement

The author wishes to thank the University of Lodz for the financial support of this research and Justyna Sierzchala, M. Sc. for performing some of the analysis.

References

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