ABSTRACT

Cystatin C, a 13 kDa low molecular weight protein is an inhibitor of cysteine proteases. Due to its low molecular weight and positive charge at physiological pH, it is freely filtered by the glomerulus and catabolized after reabsorption by proximal tubular cells with a low concentration (0.03-0.3 mg/L) in urine amongst healthy subjects. Urinary cystatin C is a potential biomarker detection of acute kidney injury (AKI) in the acute phase when patients are submitted to the intensive care unit. The aim in this report was to perform a full method validation of urinary analysis of cystatin C on a high throughput chemical analyzer by particle-enhanced turbidimetric immunoassay (PETIA) at the University Hospital in Uppsala, Sweden. The antigen excess, linearity, lower limit of quantification (LoQ), recovery, assay precision, stability and interference caused by haemoglobin was evaluated. No hook effect was observed, the assay was linear over the studied interval <0.001-0.950 mg/L with a regression of $R^2=0.9994$. The LoQ was calculated to 0.020 mg/L with a coefficient of variation (CV) $\leq$10% which was considered acceptable. The assay had a recovery between 93-100% and the assay precision had a total CV $<3.5\%$. Cystatin C is stable for 3 days in room temperature and 14 days in +4C. The assay did not show any major interference with haemoglobin. The urinary cystatin C showed good precision and performance characteristics by measurements using PETIA all of which is a necessary qualification for a biomarker at a 24-h running routine laboratory.
INTRODUCTION

The kidneys are bean-shaped organs located retroperitoneal on either side of the spine and with the right kidney generally located two centimetres lower than the left kidney. The kidney has several important functions: excretion of waste products, maintenance of extracellular fluid volume and osmolality, regulating the concentrations of ions in extracellular fluid, contributing to the acid – base balance and hormone synthesis. Each kidney holds approximately one million nephrons with each nephron consisting of capillary tufts or glomerulus, proximal tubule, loop of Henle, distal tubule and the collecting ducts. The glomerulus consists of the capillary endothelium, basal membrane and epithelial cells. This filter denies any further passage of blood cells and proteins that are >50 kDa. Proteins lower than 50 kDa is filterable, this meaning low molecular weight proteins, monosaccharides, polypeptides, amino acids and ions are freely filtered through to the proximal tubules. The ultra filtrate has a similar composition as plasma except that it is almost protein free. This liquid is known as the primary urine. Reabsorption of primary urine occurs in the proximal tubules where the remaining components, such as polypeptides, monosaccharides, amino acids, 80% sodium and potassium are reabsorbed completely. The medullary hyperosmolality allows the residual water and sodium to be reabsorbed when entering the loop of Henle. In the distal tubule, additional reabsorption of sodium and potassium is controlled by aldosterone and there is also excretion of ammonia. Subsequently the fluid is beginning to resemble the normal urine when passing the collecting ducts. Here the water is absorbed in the presence of the hormone anti diuretic hormone and concentrated urine is produced.

Acute kidney injury (AKI) is a common and serious complication highly associated with morbidity and high mortality among hospitalized patients [1,2] and is frequently occurring in hospitalized patients with an incidence of 25% in the intensive care unit [3]. AKI causes an accumulation of toxins and other nitrogenous waste products, which under normal conditions are excreted in the urine. AKI is commonly characterized as a sudden decrease in renal function and glomerular filtration rate glomerular filtration rate, resulting in major disturbance of the kidneys homeostasis and apoptosis of renal tubular cells [4,5]. AKI is seen in patients with prerenal azotemia, patients undergoing cardiothoracic surgery or cardiopulmonary bypass but the most important cause is sepsis [1,6,7]. 5% of intensive care unit patients with AKI are unfortunately forced to undergo a renal replacement therapy and data collected over the last 20 years indicate an increase of renal replacement therapy incidence [8]. Risk, injury, failure, loss and end-stage renal disease (RIFLE) and acute kidney injury network (AKIN) are two recently standardized systems of criteria for the clinical classification of AKI. But these systems are not ideal for the diagnosis of AKI as the criterion is based on elevations of the current gold standard for AKI serum creatinine [9]. Serum creatinine and other traditional markers for detection of renal failure are considered unspecific and unreliable because of its delayed response to acute renal injury. The need for novel biomarkers in the diagnosis of AKI is widely known throughout the clinical practices and intensive care units [2,7].

Cystatin C is an endogenous non glycosylated low molecular weight protein with a molecular mass of 13 kDa and a total of 120 amino acids in its polypeptide chain. Cystatin C belongs to the type II cystatin gene superfamily and is a strong inhibitor of cysteine proteases that causes proteolysis and tissue damage such as papain-like, leguamin-like cysteine protease, ficin and cathepsins to name a few. Especially, the competitive inhibition of papain-like cysteine protease by cystatin C is known as one of the most powerful in biochemistry. The superfamly of cystatins can be divided into three families: steffins, cystatins and kininogens according to their structure and cellular presence. Cystatin C is produced at a constant rate by all nucleated cells and secreted extracellularly shortly after synthesis therefore it is present in all body fluids and tissues [10-12]. The 7.3 kb gene encoding for cystatin C is localized on chromosome 20 and considered as a housekeeping-type of gene which may explain the stable protein production throughout the body. After its secretion, cystatin C is filtered freely through the glomerulus due to its positive charge at physiological pl and low molecular weight [13]. After filtration, the low molecular weight protein is reabsorbed in the proximal tubule by proximal tubular cells and almost completely catabolized and degraded in healthy individuals. The remaining cystatin C protein that is not catabolized is eliminated with the urine in very low concentrations ranging 0.03-0.3 mg/L in healthy individuals [14].

The amino acid sequence of human cystatin C was determined in 1981. At that time the biological function of cystatin C and its use in the clinic was unknown [15]. Today cystatin C is used primarily as a biomarker to estimate glomerular filtration rate and kidney function measured in serum or plasma [16]. Unlike creatinine, cystatin C is independent of sex, muscular mass and age making it a more suitable glomerular filtration rate biomarker [17,18]. Early investigations in 1985 suggested that the level of cystatin C in serum or plasma is a good predictor for estimating glomerular filtration rate

F. Hikmet Noraddin 2011
The assessment of cystatin C in other body fluids is a new and exciting field. Studies have shown cystatin C concentrations to be 0.80-2.5 mg/L in plasma, 8-14 mg/L in cerebrospinal fluid and 0.03-0.3 mg/L in urine [14].

Urinary cystatin C has recently been proposed as a biomarker for renal failure, in particular diagnosing the acute stage of kidney failure, such as AKI and acute tubular necrosis [1,11]. Urinary cystatin C has also been suggested as a marker in the assessment of glomerular filtration rate, which sounds appealing because of its non-invasive requirement [20] but there are authors who disprove the use of urinary cystatin C as a glomerular filtration rate marker [21,22]. Thus, cystatin C in urine has been used in research as a good indicator of renal disorders affecting the tubular cells first of all which is believed to be the general cause of AKI [1]. For tenofovir-treated patients with AIDS urinary cystatin C has proven useful when monitoring the renal safety in follow-up examinations [23]. It is also usable in patients suffering from haemorrhagic fever with renal implications and patients with type II diabetes with general nephropathy [24,25]. These findings may indicate urinary cystatin C as a broad marker for tubular damage in patients with varied tubular kidney complications. The absence of circadian variations simplifies the interpretation of test results and allows intensive care unit personal a fast and reliable estimation of renal damage [26].

In this study the assay performance of urinary analysis of cystatin C was evaluated with particle enhanced turbidimetric immunoassay (PETIA) with yolk antibodies (IgY). IgY, or chicken antibodies, is the major serum immunoglobulin in chickens and other avian species. IgY is transported to the egg from the hen in a similar way as mammalian IgG crosses the placenta barrier. The concentration of IgY is high in egg yolk and very low in egg white [27]. Avian antibodies are phylogenetically different from the mammalian antibodies, which gives them several advantages when used in immunoassays. IgY does not activate the complement system and does not cross-react with rheumatoid factors in patients with rheumatoid arthritis, human anti-murine antibodies in patients treated with mouse monoclonal antibodies and bacterial Fc receptors [27,28]. IgY is similar to mammalian IgA when comparing structure and molecular weight whereas IgY molecular weight has been reported to be approximately 167 kDa with a larger heavy chain and smaller light chain compared to IgG [29]. However, IgY differs in antibody diversity due to the rearrangement of the light chain [28].

Finally, the aim of this study was to validate the assay performance of urinary analysis of cystatin C on a fully automated high throughput turbidimetric analyzer for routine diagnostic and clinical use, at the University Hospital in Uppsala, Sweden.

**MATERIALS AND METHODS**

**Samples**

Samples were collected from the routine at the Department of Clinical Chemistry and Pharmacology, Uppsala University Hospital. Human urine samples with varying concentrations of cystatin C were found by searching urine samples with high albumin concentrations (≥100 mg/day) in the laboratory computer system FLEXLAB. Samples were always decoded before work commenced. Urine samples were centrifuged at 2400 g x 5 min. The supernatant was transferred to a new tube and the pellet was discarded. The samples were measured in single initially to determine the concentration of cystatin C. To achieve samples with a low cystatin C concentration, samples were either diluted with saline (0.9% NaCl) or pooled with urine samples containing low concentrations of cystatin C.

**Urinary Cystatin C assay**

Reagent 1 assay buffer (REF 1101), reagent 2 immunoparticles (REF 1101), calibrator (REF 1012) with a concentration of 7.8 mg/L and purified delipidated human serum Cystatin C with a concentration of 35 mg/L were all provided by Gentian AS (Moss, Norway). Two levels of controls were used, BioRAD Seronorm level 1 (diluted 1:6) and BioRAD Immunoassay Liquicheck (Hercules, CA, USA). Before analyzing, controls and calibrator were put on a slow blender for 3-5 min.

The method is based on a PETIA technique. Immunoparticles consists of purified avian antibodies (IgY) directed to human cystatin C that are covalently attached on uniform polystyrene particles. The cystatin C solution is ready to use and is preserved with 15 mmol/L sodium azide and antibiotics. The assay buffer contains of a 3-(N-morpholino)-propanesulfonic acid buffered saline, preserved with sodium azide. Urinary cystatin C measurements were performed on Abbott Architect ci8200 analyzer (Abbott Park, IL, USA). 10 µL of sample and 180 µL assay buffer was mixed in a cuvette and incubated around 5 min. 45 µL immunoparticles was added at point 16 (each photometric point is 18 seconds) and incubated.
around 5 min. The immune complexes formed by agglutination were measured at 444 nm and the absorbance was proportional to the concentration of cystatin C expressed in mg/L. The calibration was set at six points and run automatically in duplicates covering the range 0.042-1.050 mg/L. Test results over the highest calibrator point were simply displayed as >1.050 mg/L and were automatically rerun on the instrument with a 1:8 dilution, using saline solution contained in the instrument. Further instrument settings were as follows: Absorbance blank at point 19, main absorbance measure at point 31-33, and spline calibration method. Test results were reported in mg/L with 3 decimals.

**Assay precision**

The assay precision was evaluated by three patient samples with different levels of cystatin C: 0.13 mg/L, 0.57 mg/L and 0.90 mg/L. The samples were divided into 10 equal portions with a total volume of 700 μL and frozen at -20 C. Before each run the assay was calibrated and controls were analyzed. Each sample was thawed in a +37 C water bath, vortex mixed and measured in duplicate twice a day with at least 2 h between each run. For each sample within-run coefficient of variation (CV), between-run CV, between-day CV and total CV was calculated.

**Lower limit of quantification (LoQ)**

LoQ study was performed to determine the lowest actual amount of cystatin C in urine that could be reliably detected with a CV ≤10%. A sample pool with cystatin C concentration of 0.50 mg/L was determined by measuring in duplicate. Sample dilutions were made (1:5, 1:10, 1:15 and 1:20) with saline. The diluted samples were run in 5 replicates, 4 times with 1 h between each run. Within each run controls were measured in singles.

**Analyte stability**

Five urine samples were divided into 7 portions in which, 3 portions were stored at room temperature and 4 portions were stored in +4 C. All samples were measured initially, at day 3 and 7 and one additional sample that had been stored in +4 C was measured at day 14. The assay was calibrated before each run. Controls and samples were measured in duplicates. The percentage change in cystatin C concentration was calculated, using Excel 2007.

**Antigen excess**

The antigen excess was determined to identify the critical hook concentration. A dilution series of spiked purified serum cystatin C with a concentration of 35 mg/L was prepared by diluting the stock solution from 35 mg/L to 0.436 mg/L. The sample absorbance values were plotted against the calculated theoretical sample concentrations.

**Assay recovery**

Recovery was performed to evaluate and determine the dilution error in percent. Purified serum cystatin C (35 mg/L) was diluted with saline to a concentration of 5 mg/L and was used as stock solution. 2.5%, 5% and 10% dilutions of the stock solution were made and analyzed in triplicates to determine the concentration. A pooled urine sample at 0.156 mg/L was determined in triplicate. The urine sample was spiked with the stock solution matching the theoretical calculated concentrations at 0.156, 0.300, 0.444, 0.588, 0.732, 0.877 and 1.021. Samples were measured in duplicates and the difference between calculated and measured values was expressed in percent. A recovery ±10% was considered acceptable.

**Haemoglobin interference**

A hemolysate was provided by Gentian. Erythrocytes were centrifuged and washed with cold isotonic saline 4 times. The erythrocytes were lysed by adding distilled water and frozen down. The cellular particles were separated with centrifugation. The haemoglobin concentration was determined with a haemoglobin assay with a concentration of 57 g/L. A high and a low cystatin C sample with concentrations of 1.0 mg/L and 0.1 mg/L respectively were measured 10 times. The CV for these 10 replicates was used to determine the number of replicates further in the study. The high sample and the low sample were mixed with hemolysate to gain a volume of 5 mL with a final haemoglobin concentration of 10 g/L. An additional 5 mL of the high and the low samples were mixed preparing a control by adding the same volume saline as volume hemolysate. Samples were measured in 4 replicates and CV and percentage change were calculated.

**Assay linearity**

A pooled sample A, with a high cystatin C concentration (0.953 mg/L) and a pooled sample B, with almost no detectable concentration of cystatin
C were pooled and determined by analyzing in triplicate. A dilution series was made by adding different portions of the high sample and the low sample (A10+ B0, A9+B1, A8+B2 ... A0+B10). Linearity was evaluated by plotting the measured values of sample pools run in duplicates, against the theoretical values.

RESULTS

Assay precision

Table 1. The mean value and CV of three urinary cystatin C samples at different levels.

<table>
<thead>
<tr>
<th>Cystatin C level mean (mg/L)</th>
<th>0.129</th>
<th>0.568</th>
<th>0.898</th>
</tr>
</thead>
<tbody>
<tr>
<td>Within-run CV%</td>
<td>1.64</td>
<td>0.71</td>
<td>0.35</td>
</tr>
<tr>
<td>Between-day CV%</td>
<td>1.27</td>
<td>1.89</td>
<td>1.76</td>
</tr>
<tr>
<td>Between-run CV%</td>
<td>2.11</td>
<td>2.49</td>
<td>1.98</td>
</tr>
<tr>
<td>Total CV%</td>
<td>2.96</td>
<td>3.20</td>
<td>2.67</td>
</tr>
<tr>
<td>n=30</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The assay precision was determined by calculating CV for 3 cystatin C samples. The obtained CV values did not exceed 3.5% at any level (Table 1). Controls were also analyzed; the high control had a total CV below 3.5% (2.89%) and the low control had a total CV of 8.27% (data from control measurements are not shown here).

Lower limit of quantification (LoQ)

Table 2. The total CV% and mean value of urine cystatin C sample dilutions made with saline.

<table>
<thead>
<tr>
<th>Dilution factor</th>
<th>1:5</th>
<th>1:10</th>
<th>1:15</th>
<th>1:20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample mean (mg/L)</td>
<td>0.100</td>
<td>0.045</td>
<td>0.028</td>
<td>0.020</td>
</tr>
<tr>
<td>Total CV%</td>
<td>1.97</td>
<td>3.91</td>
<td>6.28</td>
<td>8.76</td>
</tr>
</tbody>
</table>

The LoQ was determined by measuring urine sample dilutions made with saline in 4 runs, using 5 replicates in each run. All results showed a CV <10% which did not exceed the limit and was considered acceptable (Table 2).

Haemoglobin interference

The samples and controls were measured in 4 replicates and single, respectively. The low cystatin C sample without hemolysate had a concentration mean of 0.078 mg/L and the high sample had a mean of 0.722 mg/L. The low sample with hemolysate had a loss in cystatin C concentration by 8.0% (CV 17.1%). The high sample increased in cystatin C concentration with 12.3% (CV 0.31%).

Analyte stability

All stored samples in +4C resulted in a loss of cystatin C concentration after 14 days ranging between -0.6 to -11.0% (Figure IA and B). The samples stored in room temperature gave a varied result with both increasing and decreasing concentrations of cystatin C after 7 days. For 4 samples the result ranged from -11.1 to +9.0%. One sample (Δ) decreased drastically after 7 days and had a 51.0% loss of cystatin C concentration from 0.204 to 0.100 mg/L (Figure IB).
Figure 2. Increasing theoretical concentrations of cystatin C plotted against the observed delta absorbance values (●). The calibration standard curve (▲) is also plotted and the dashed line represents the 6th standard value + 10% which is the cut-off value for antigen excess.

Antigen excess

There was no hook effect observed at any concentration ranging from 0.436 to 35 mg/L with samples measured in duplicates run on the instrument (Figure 2). Test result that exceeded the highest calibration point generated a result of >1.050 mg/L. The absorbance values were retrieved from the instrument software for each measurement.

Assay recovery

Table 3. The calculated sample recovery from a dilution series. The dilution series were made with a pooled cystatin C (0.156 mg/L) and purified serum cystatin C (5 mg/L).

<table>
<thead>
<tr>
<th>Recovery</th>
<th>Theoretical concentration</th>
<th>Mean observed value</th>
<th>CV%</th>
<th>Recovery%</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.156</td>
<td>0.155</td>
<td>0.00</td>
<td>99.6</td>
</tr>
<tr>
<td>1</td>
<td>0.300</td>
<td>0.280</td>
<td>0.51</td>
<td>93.3</td>
</tr>
<tr>
<td>2</td>
<td>0.444</td>
<td>0.422</td>
<td>0.17</td>
<td>94.9</td>
</tr>
<tr>
<td>3</td>
<td>0.588</td>
<td>0.584</td>
<td>0.39</td>
<td>93.1</td>
</tr>
<tr>
<td>4</td>
<td>0.732</td>
<td>0.687</td>
<td>0.52</td>
<td>93.8</td>
</tr>
<tr>
<td>5</td>
<td>0.877</td>
<td>0.824</td>
<td>0.51</td>
<td>93.9</td>
</tr>
<tr>
<td>6</td>
<td>1.021</td>
<td>0.970</td>
<td>0.00</td>
<td>95.0</td>
</tr>
</tbody>
</table>

The recovery ranged from 93 to 100% for the 7 prepared dilutions which covered the calibrator range, with a CV ≤ 0.5% at all levels (Table 3). The 2.5%, 5% and 10% stock solution dilutions with saline gave a mean value of 5.27 mg/L when multiplying the dilution factor for each measurement.

Assay linearity

The assay showed good linearity and the assay linearity equation was \( y = 1.0125x - 0.0065 \) with a regression of \( R^2 = 0.9994 \) over the studied interval >0.001 mg/L to 0.950 mg/L (Figure 3).

DISCUSSION

Cystatin C is a 13 kDa low molecular weight protein that is produced at a constant rate by all nucleated cells with a stable secretion and is present in almost all bodily fluids and tissues [10]. In healthy individuals, cystatin C is excreted in very low concentrations by urine. This means that an increased amount of cystatin C in urine may indicate an injury to the tubular cells [11].

In the current situation, cystatin C is an established biomarker for glomerular filtration rate estimating measurements in plasma or serum to detect glomerulus kidney abnormalities [16]. In the past few year measurements of cystatin C in urine has attracted attention as a potential biomarker to discover AKI or acute tubular necrosis and also other pathological changes of the kidney tubules [1, 24]. Thus, the aim of this study was to validate such a biomarker with a potential future capacity of detecting tubular damage with good precision and performance on a high throughput chemical analyzer that may be useful in the acute phases of patients who are submitted to the intensive care unit with renal implications.

All three levels of cystatin C showed low imprecision with an acceptable CV <3.5% which
complies with results reported by Herget-Rosenthal with co-workers and Conti with co-workers. In these studies, the used method for measuring cystatin C was particle-enhanced nephelometric immunoassay (PENIA) [10,11]. Earlier publications have shown that differences between PETIA and PENIA are very small [30,31]. Accordingly, the LoQ study confirmed the assay precision study but it showed a slightly lower CV at similar concentrations of cystatin C. This phenomenon may be explained because of the many substantial factors affecting the precision study as the between-day variance, between-run variance, the calibrator batch-to-batch differences and handling of samples with different starting points etc. The LoQ result allows precise measurements at low concentrations of cystatin C. However, is that really necessary from a clinical perspective to measure cystatin C at detection level for selecting patients with renal tubular implications? In healthy subjects, the concentration of cystatin C is low (0.03-0.3 mg/L) and is within the studied concentration interval chosen for the LoQ study. From an assay performance point of view, this is additional evidence that the assay is a good and accurate method. The stability of cystatin C in urine is acceptable after 3 days and 14 days in room temperature and +4C, respectively. This allows urine samples to be shipped from health centers and nearby health clinics to the laboratory by mail at room temperature, and after analysis store samples in +4C in case a reanalysis is required. There are also daily transports to the laboratory from nearby health clinics and test results are usually reported at the same day after sample arrival at the laboratory. The stability of cystatin C in urine is thereby enough for the laboratory when the assay is eventually included in the routine. However, one sample stored at room temperature resulted in a heavy decrease of cystatin C. We have no explanation regarding the decrease but a theoretical explanation might possibly be bacterial contamination. Another theory could be presence of proteolytic enzymes in the urine [10]. The same sample stored at +4C did not show similar tendency and that may imply that the exception only occurred on the sample stored in RT. In a study by Conti with co-workers the collection of urine samples was done by adding a cocktail containing antiprotease and antimicrobial agent. They reported a decrease in samples without the cocktail [10]. Nevertheless, we have shown that cystatin C in urine is stable for 3 days in room temperature (Figure 1A and B). Further studies in RT could be done to evaluate the stability from day 3 to 7 with added antiprotease and antimicrobial agents to possibly extend the stability. There was no hook effect observed for the assay. In healthy individuals, the concentration of cystatin C in urine is low as stated before but in patients suffering of severe renal tubular disorders the concentration can lead to a 200-fold increase [13]. The assay will not report incorrect test result up to 35 mg/L because none of the samples fell below the dashed line (Figure 2) which represents the cut-off value of antigen excess. Further, the assay recovered within the accepted limit ±10%. Samples with concentrations over the highest calibration point were automatically rerun with a 1:8 dilution on the instrument and based on the assay recovery, test results can be accepted. Haemoglobin did not interfere at a clinical significance. Testing the cystatin C-haemoglobin interference at such a high concentration as 10 g/L haemoglobin should give a satisfactory margin for analyzing urine samples from patients with heavy haematuria. The assay was linear over the calibration interval with good correlation and intercept.

In this study, IgY was used to measure cystatin C in urine. IgY does not cross-react with rheumatoid arthritis factors, complement, human anti murine antibodies or bacterial Fc-receptors due to its phylogenetic differences [32]. The advantages over mammalian immunoglobulins are many and the IgY molecule is slightly different to IgG. IgY does not have the flexible hinge region as mammalian IgG. Studies have however shown that IgY is more suitable for turbidimetric measurements and gives a better analytical performance [30,33]. IgY often binds to other epitopes on the antigen compared to mammalian IgG and produces antibody diversity in a different way. IgY is found in high concentrations in egg yolk and the recovery of antibody is far greater after purification from egg yolk than after antibody purification from mammalian sources. This may allow a reduction in the antigen proportion used for immunization [32].

The urinary cystatin C turbidimetric immunoassay is a rapid and easy method for fast measurements with short turnaround time. A full validation was made at the Department of Clinical Chemistry and Pharmacology, University Hospital using the same reagents as the already established plasma method. Recently, the laboratory also validated the assay cerebrospinal fluid cystatin C [34].

In summary, the use of urinary analysis of cystatin C is at a final research stage and more studies must be done before it can reliably be introduced to the routine. Several publications have reported good results of cystatin C as a biomarker being of importance for the diagnosis of AKI in the acute stage of renal tubular damage [1] and monitoring tubular function in patients after kidney transplantation and patients taking nephrotoxic medication [35,23].
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