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## Determination of norfloxacin in human urine by capillary electrophoresis with electrochemiluminescence detection

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**Abstract** A fast and sensitive approach that can be used to detect norfloxacin in human urine using capillary electrophoresis with end-column electrochemiluminescence (ECL) detection of  $Ru(bpy)_3^{2+}$  is described. The separation column was a 75- $\mu\text{m}$  i.d. capillary. The running buffer was 15  $\text{mmol L}^{-1}$  sodium phosphate (pH 8.2). The solution in the detection cell was 50  $\text{mmol L}^{-1}$  sodium phosphate (pH 8.0) and 5  $\text{mmol L}^{-1}$   $Ru(bpy)_3^{2+}$ . The ECL intensity varied linearly with norfloxacin concentration from 0.05 to 10  $\mu\text{mol L}^{-1}$ . The detection limit (S/N=3) was 0.0048  $\mu\text{mol L}^{-1}$ , and the relative standard deviations of the ECL intensity and the migration time for eleven consecutive injections of 1.0  $\mu\text{mol L}^{-1}$  norfloxacin ( $n=11$ ) were 2.6% and 0.8%, respectively. The method was successfully applied to the determination of norfloxacin spiked in human urine without sample pretreatment. The recoveries were 92.7–97.9%.

**Keywords** Capillary electrophoresis · Electrochemiluminescence · Norfloxacin · Tris(2,2'-bipyridyl) ruthenium(II) · Urine

### Introduction

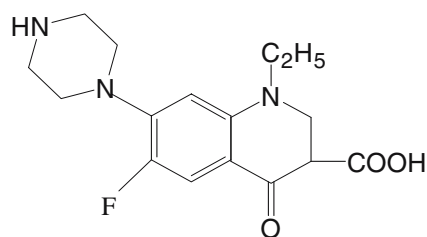
Norfloxacin [chemical name: 1-ethyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinolinecarboxylic acid] (Fig. 1) was the first modern fluoroquinolone to be made

commercially available [1]. Norfloxacin is an excellent broad-spectrum synthetic pharmacokinetic antibacterial agent [2]. Since its development, within the last decade, the compound has been widely applied in clinical treatment. Norfloxacin is active against many gram-negative and gram-positive bacteria, including gentamicin-resistant *Pseudomonas aeruginosa* and methicillin-resistant *Staphylococcus aureus* [3]. The bactericidal action of norfloxacin results from interference with enzyme DNA gyrase, which is needed for the synthesis of bacterial DNA [4, 5]. Excellent therapeutic effects have been shown in the treatment of urinary and respiratory tract infections, gastro-intestinal and sexually transmitted diseases with norfloxacin [6, 7]. About 30% of the norfloxacin dose is excreted unchanged in the urine within 24 h, and it is necessary to monitor relatively concentrated levels of urinary norfloxacin. Many analytical methods, including spectrophotometry [8–10], fluorimetry [11–14], high-performance liquid chromatography (HPLC) with various detection systems [15–24] and electrochemistry [25], have been reported for the determination of norfloxacin. These methods require expensive HPLC columns and costly apparatus such as LC-MS and/or extraction procedures that use toxic organic solvents. In addition, the analysis times of these methods are relatively long.

The capillary electrophoresis (CE) technique has been around for over 20 years. CE is an important and powerful analytical tool [26]. The advantages of CE include small sample size, high resolution, short analysis time, and low operational cost [27]. However, sensitive detection methods, such as laser-induced fluorescence (LIF), are required because of the small sample volume. LIF is a relatively costly detection system [28]. Electrochemiluminescence (ECL) detection with  $Ru(bpy)_3^{2+}$  is a viable alternative for CE detection. ECL has the advantages of simplicity, inexpensive instrumentation, low background noise, high sensitivity, good selectivity, and wide dynamic linear range [27]. ECL has been applied to a variety of fields such as biosensors, immunoassays, and flow injection analysis [29]. CE with ECL detection using  $Ru(bpy)_3^{2+}$  was investigated in the mid-to-late 1990s as a method of

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**Fig. 1** Molecular structure of norfloxacin

determining a variety of analytes [30–38]. In this work, capillary electrophoresis with ECL detection is applied to the determination of norfloxacin in human urine. The urine sample was assayed directly without pretreatment. The method is highly sensitive, simple and reliable.

## Experimental

### Reagents and chemicals

All reagents used were of analytical grade. Double-distilled water (DDW) was used throughout. Norfloxacin was obtained from the National Institute for The Control of Pharmaceutical and Biological Products (Beijing, China). Tris(2,2'-bipyridyl) ruthenium(II) chloride hexahydrate was purchased from Alfa Aesar (Johnson Matthey, Ward Hill, MA, USA) and used without further purification. All solutions were prepared with DDW and stored in a refrigerator at 277 K. Working standard solutions were freshly prepared by precise dilution of stock solutions with DDW. The buffer pH was adjusted with orthophosphoric acid or sodium hydroxide (Beijing Chemical Company, Beijing, China). Prior to CE analysis, the required sample solutions and phosphate buffer were filtered through 0.45- $\mu\text{m}$  membrane filters (Shanghai Xinya Purification Material Factory, Shanghai, China).

### Apparatus

The CE–ECL system (a high-voltage power supply, an electrical potentiostat, a multifunction chemiluminescence detector and a multichannel data collection analyzer) was produced by Xi'an Remex Electronic Science-Tech Co., Ltd. (Xi'an, China). The output ECL intensity was amplified and recorded on a Pentium 4 PC using the MPI-B software.

The CE–ECL detection cell has been described previously [35]. The end-column ECL cell was composed of a three-electrode system, with a 300  $\mu\text{m}$ -diameter Pt disk as the working electrode, a Pt wire as the counter electrode, and a Ag/AgCl electrode as the reference electrode. The surface of the working electrode was polished with 0.3  $\mu\text{m}$  alumina powder and cleaned with water in an ultrasonic cleaner before use. A reactivation process to eliminate the oxide layer on the Pt electrode was performed by scanning the applied potential on the Pt disk from  $-0.5$  V to 0.0 V

(vs. Ag/AgCl) for ten cycles. The performance of the working electrode was stable for at least two months after the electrochemical treatment. Approximately 300  $\mu\text{L}$  of  $\text{Ru}(\text{bpy})_3^{2+}$  (5 mmol  $\text{L}^{-1}$ ) in phosphate buffer (50 mmol  $\text{L}^{-1}$ , pH 8.0) was placed in the ECL detection cell for ECL measurement. The  $\text{Ru}(\text{bpy})_3^{2+}$ –phosphate solution was replaced every 2 h during the experiments to avoid errors in ECL measurement due to possible changes in  $\text{Ru}(\text{bpy})_3^{2+}$  concentration. All electrophoretic experiments were performed with a 40-cm uncoated silica capillary (75  $\mu\text{m}$  i.d., 375  $\mu\text{m}$  o.d.) (Yongnian Optical Fiber Co., Hebei, China). The capillary was conditioned before use by flushing the capillary with 0.1 mol  $\text{L}^{-1}$  sodium hydroxide for 24 h followed by rinsing with distilled water for 30 min. The capillary was rinsed with water and running buffer for 5 min, respectively, between CE runs.

### Sample preparation

Norfloxacin stock standard solution (0.1 mmol  $\text{L}^{-1}$ ) was prepared in DDW. This solution was stable for at least one week when stored in the dark at 277 K. Working solutions were prepared by diluting the solution with water just before use.

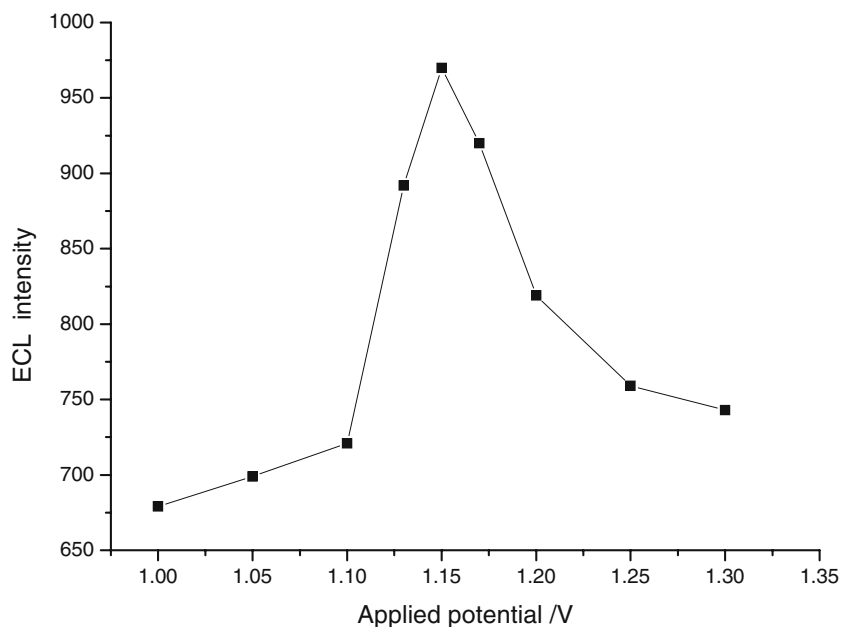
Fresh urinary samples were taken from a healthy student volunteer in the laboratory and were filtered through a membrane (0.45  $\mu\text{m}$ ). After that, the samples were diluted with water. The filtrate was injected directly by applying 12 kV to the CE–ECL system for 6 s. Electrophoresis was performed at 15 kV.

## Results and discussion

### Optimization of detection conditions

The electrochemiluminescence (ECL) intensity is dependent on the rate of the light-emitting chemical reaction, which is in turn dependent on the potential applied to the electrode. Figure 2 shows the ECL intensity of norfloxacin versus an applied potential of 1.00 V to 1.35 V (vs. Ag/AgCl). At an applied potential of less than 1.00 V, ECL emission was not observed since  $\text{Ru}(\text{bpy})_3^{2+}$  was not oxidized on the electrode. The ECL intensity of norfloxacin increased as the potential applied was increased from 1.00 to 1.15 V. The ECL intensity reached its maximum value at 1.15 V. Above 1.15 V, the light intensity decreased significantly. Clearly, the optimum potential for ECL is 1.15 V, corresponding to the oxidation potential of  $\text{Ru}(\text{bpy})_3^{2+}$ . The oxidation of  $\text{Ru}(\text{bpy})_3^{2+}$  played an important role in the ECL reaction of norfloxacin [39]. In the following experiments, the voltage applied was maintained at 1.15 V (versus Ag/AgCl). Under this condition,  $\text{Ru}(\text{bpy})_3^{2+}$  was oxidized to  $\text{Ru}(\text{bpy})_3^{3+}$  to a large extent, and the baseline of ECL measurement was stable.

**Fig. 2** Hydrodynamic voltammogram for norfloxacin in phosphate buffer



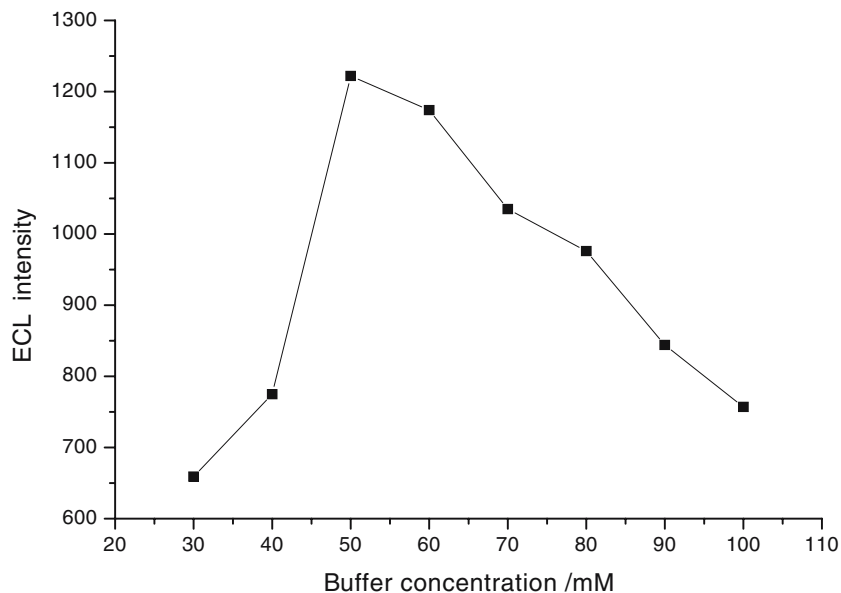
The sample was  $10 \mu\text{mol L}^{-1}$  norfloxacin. Electrokinetic injection of the sample was used; the injection lasted 8 s and a voltage of 10 kV was applied. The CE separation voltage was 15 kV. Separation buffer was  $10 \text{mmol L}^{-1}$  phosphate buffer at pH 8.0. The solution in the ECL cell was  $5 \text{mmol L}^{-1} Ru(bpy)_3^{2+}$  in  $80 \text{mmol L}^{-1}$  phosphate buffer (pH 8.2).

The optimal concentration of  $Ru(bpy)_3^{2+}$  in the detection cell is one of the most important detection parameters. A low concentration of  $Ru(bpy)_3^{2+}$  leads to a low background noise. Detection sensitivity increases with the concentration of  $Ru(bpy)_3^{2+}$ . The background noise, however, also increases with the concentration of  $Ru(bpy)_3^{2+}$ , and a larger amount of the expensive reagent is consumed too.

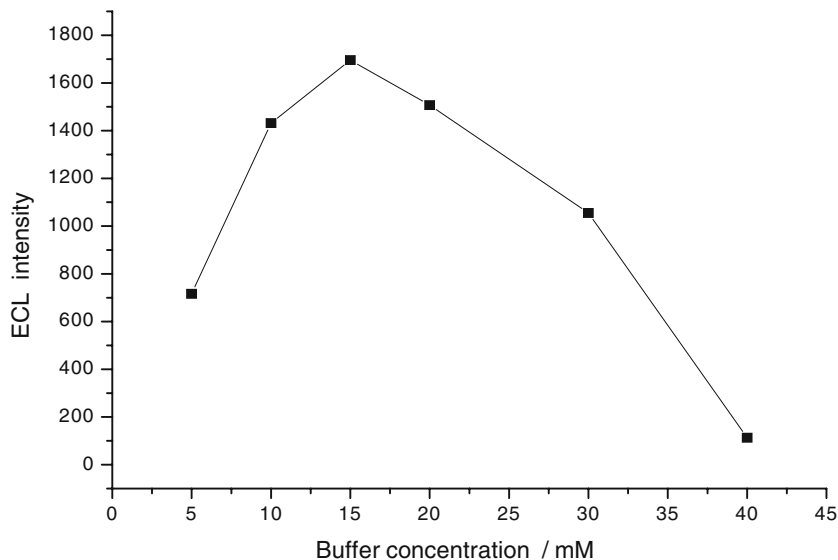
In order to achieve a relatively high ECL intensity with moderate reagent consumption,  $5 \text{mmol L}^{-1} Ru(bpy)_3^{2+}$  was used in this study. The solution was replaced after 2 h of measurement in order to maintain good reproducibility of the ECL measurements.

The concentration of the buffer in the detection cell affects the ECL intensity. Figure 3 shows a plot of ECL intensity versus the concentration of the buffer from  $10 \text{mmol L}^{-1}$  to  $100 \text{mmol L}^{-1}$  (pH 8.2). The experimental conditions were similar to those of Fig. 2. The potential applied to the Pt electrode was 1.15 V. The highest ECL intensity was obtained at buffer concentration of  $50 \text{mmol L}^{-1}$ . A buffer concentration of  $50 \text{mmol L}^{-1}$  was used in the following experiments.

**Fig. 3** ECL intensity of norfloxacin versus the concentration of the phosphate buffer in the ECL cell



**Fig. 4** The effect of the concentration of the running phosphate buffer on the ECL intensity



Determination conditions: sample  $10 \mu\text{mol L}^{-1}$  norfloxacin; separation capillary:  $75 \mu\text{m}$  i.d. and  $40 \text{ cm}$  length; electrokinetic injection for  $8 \text{ s}$  at  $10 \text{ kV}$ ; separation voltage  $15 \text{ kV}$ ; detection potential  $1.15 \text{ V}$ ; separation buffer  $10 \text{ mmol}\cdot\text{L}^{-1}$  phosphate buffer at  $\text{pH } 8.0$ ; in ECL cell:  $5 \text{ mmol L}^{-1} \text{Ru}(\text{bpy})_3^{2+}$ , phosphate buffer at  $\text{pH } 8.2$ .

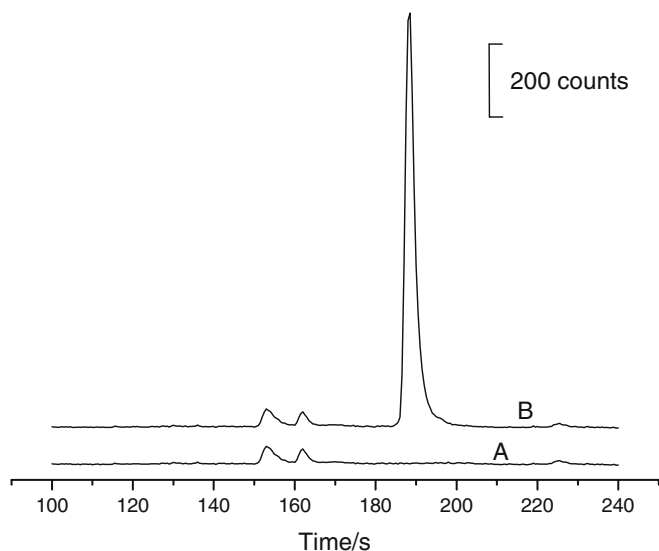
Previous reports indicated that the ECL emission intensity was markedly correlated with the analyte  $\text{p}K_{\text{a}}$  [40–42]. The deprotonation step of the analyte in the ECL reaction is an important factor that affects the ECL intensity. The effect of the buffer pH on the ECL intensity of norfloxacin was investigated over the pH range of 6–9. When the pH value was 8.0, the ECL intensity reached the maximum value. ECL intensity then decreased with increasing buffer pH. The optimized pH value for this study was 8.0.

#### Optimization of separation conditions

The effects of the concentration of the phosphate buffer on the migration time and the ECL intensity of norfloxacin were investigated. The pH of the CE buffer was maintained at 8.2. The concentration of the phosphate buffer was 5–50  $\text{mmol L}^{-1}$ . The experimental conditions were similar to those of Fig. 3. The concentration of the phosphate buffer in the ECL detector was  $50 \text{ mmol L}^{-1}$ . The ECL intensity initially increased with increasing running buffer concentration (Fig. 4). The highest ECL intensity was obtained at a running buffer concentration of  $15 \text{ mmol L}^{-1}$ . At buffer concentrations above  $15 \text{ mmol L}^{-1}$ , the ECL intensity decreased and the ECL baseline became unstable. The unstable baseline may be due to interference in the ECL detector from the increased electrophoretic current. In the following experiments,  $15 \text{ mmol L}^{-1}$  phosphate buffer was used as the CE running buffer.

Determination conditions: sample  $10 \mu\text{mol L}^{-1}$  norfloxacin; separation capillary:  $75 \mu\text{m}$  i.d. and  $40 \text{ cm}$  length; electrokinetic injection for  $8 \text{ s}$  at  $10 \text{ kV}$ ; separation voltage  $15 \text{ kV}$ ; detection potential  $1.15 \text{ V}$ ; separation buffer was phosphate buffer at  $\text{pH } 8.0$ ; in ECL cell:  $5 \text{ mmol L}^{-1} \text{Ru}(\text{bpy})_3^{2+}$ ,  $50 \text{ mmol L}^{-1}$  phosphate buffer at  $\text{pH } 8.0$ .

The pH of the CE buffer plays an important role in CE separation and the ECL intensity [43]. The pH of the CE buffer determines the electroosmotic flow as well as the charge of the analytes, which collectively affect the



**Fig. 5a,b** Electropherograms of (a) blank urine and (b) the spiked urine

**Table 1** Determination of norfloxacin in human urine samples ( $n=5$ )

Concentration ( $\mu\text{mol/L}$ )	Recovered ( $\mu\text{mol/L}$ )	Recoveries (%)	RSD (%)
0.5	0.477	95.4%	4.2%
1.0	0.932	93.2%	6.2%
2.5	2.318	92.7%	5.7%
5.0	4.895	97.9%	5.2%

migration time. The ECL intensity of norfloxacin increased as the pH of the running buffer was increased from pH 6 to 8.2. When the buffer pH exceeded 8.2, the ECL intensity decreased and the migration time increased. This was due to the effect of the increase in the ionic strength, which resulted in an increase in Joule heating. Maximum ECL intensity and an appropriate migration time were obtained at pH 8.2. Therefore, the optimum pH value of the running buffer was taken to be 8.2.

#### Linearity, detection limit and reproducibility

The optimized experimental conditions were 40-cm separation capillary (75  $\mu\text{m}$  i.d.), 1.15 V applied potential on the Pt disc of the ECL detector cell, 5  $\text{mmol L}^{-1}$   $\text{Ru}(\text{bpy})_3^{2+}$  and 50  $\text{mmol L}^{-1}$  phosphate buffer (pH 8.0) in the detection cell, 15 kV separation voltage, 6 s injection time, 12 kV injection voltage, and 15  $\text{mmol L}^{-1}$  running buffer (pH 8.2). The calibration curve for norfloxacin was linear over the concentration range 0.05–10  $\mu\text{mol L}^{-1}$  with a regression curve of  $y=265.2x+2718$  ( $r^2=0.9935$ ). The detection limit was 0.0048  $\mu\text{mol L}^{-1}$  with a signal-to-noise ratio of 3. The relative standard deviations (RSD) of the ECL intensity and the migration time for eleven consecutive injections of 1.0  $\mu\text{mol L}^{-1}$  norfloxacin were 2.6% and 0.8%, respectively.

#### Application to human urine

The determination of norfloxacin in human urine samples was performed. Fresh urine samples from a healthy volunteer were collected in the laboratory and were filtered with a 0.45  $\mu\text{m}$  membrane. As reported earlier, the ECL intensity was strongly influenced by the matrix [44]. In this experiment, the urine sample was diluted and injected directly into the electrophoresis system for analysis. The optimized experimental conditions were used. The electropherogram for blank urine is shown in Fig. 5a. Two peaks appeared in the electropherogram. The migration of the spiked norfloxacin was slower than those of the interferences (Fig. 5b). Since the interferences were well separated from the peak of norfloxacin, little matrix effect was expected. In order to further verify the feasibility of the CE–ECL method, the proposed CE–ECL method was employed for the determination of norfloxacin in human urine samples. Five healthy volunteers were treated simultaneously with oral administration of 200 mg norfloxacin capsules. The urine samples were collected 1.5 h after oral administration of the drug. The urine collected before dosing was employed as a blank. All fresh urinary samples were filtered with a 0.45  $\mu\text{m}$  membrane and measured by the CE–ECL system. The ECL peaks obtained were similar to those seen in Fig. 5. The detection of norfloxacin did not encounter interference from the real samples. The content of norfloxacin in a urine sample was 5.23 mg/l on average. This result could be explained by many factors. For example, the volume of water imbibed,

the ability to absorb the norfloxacin, and the metabolization of norfloxacin, may not have been exactly the same for all of the healthy volunteers. There was no interference in the ECL detection of norfloxacin in the urine. The CE–ECL technique was therefore found to be applicable to the detection of norfloxacin in real urine samples.

To determine the recovery of the detection method, a standard solution of norfloxacin was added to the diluted urine solution and assayed. The samples were analyzed directly without any extraction procedures. Using the optimized experimental conditions for norfloxacin detection, the recovery of the present system for norfloxacin present at levels of 0.5–5  $\mu\text{mol L}^{-1}$  is summarized in Table 1. The recoveries were estimated by measuring the peak area of norfloxacin from the human urine sample spiked with known concentration of norfloxacin to that of the norfloxacin added to the blank urine. The relative standard deviations of the ECL intensities were 6.2% or less. The recoveries ( $N=5$ ) for norfloxacin were between 92.7% and 97.9%. Obviously, the norfloxacin was separated from the interferences in the urine. The method also has good detection selectivity. Hence, we concluded that the CE–ECL technique was useful for the determination of norfloxacin in urine samples.

#### Conclusions

A new analytical procedure based on CE–ECL has been developed for the direct determination of norfloxacin in human urine. The proposed method was simple, quick, economical and sensitive. Norfloxacin can be determined in a urine sample within 210 s. The method was successfully applied to the determination of norfloxacin spiked in human urine without pretreatment.

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