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Determination of caprolactam and 6-aminocaproic acid in human urine using hydrophilic interaction liquid chromatography-tandem mass spectrometry

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ABSTRACT

A simple and rapid assay based on hydrophilic interaction liquid chromatography with tandem mass spectrometry has been first developed and validated for simultaneous determination of caprolactam (CA) and 6-aminocaproic acid (6-ANCA) in human urine using 8-aminocaprylic acid as internal standard. A 20 μL aliquot of urine was injected directly into the liquid chromatography tandem mass spectrometry (LC–MS–MS) system. The analytes were separated on a Phenomenex Luna HILIC column with gradient elution. Detection was performed on Triple Quadrupole LC–MS in positive ions multiple reaction monitoring mode using electrospray ionization. The calibration curves were linear ($r^2 \geq 0.995$) over the concentration range from 62.5 to 1250 ng/mL for CA and 31.25 to 1000 ng/mL for 6-ANCA. The detection limits of CA and 6-ANCA were 62.5 and 15.6 ng/mL, respectively. The intra-day and inter-day precisions were within 8.7% and 9.9%, respectively. The intra-day and inter-day accuracy were between 5.3% and 3.5%, and between 6.1% and 6.6%, respectively. The method proved to be simple and time efficient, and was successfully applied to evaluate the kinetics of caprolactam in one unusual case of caprolactam poisoning.

1. Introduction

Caprolactam (CA) is an organic compound with the formula $(CH_2)_5C(O)NH$. It is used as a raw material in the manufacture of synthetic Perlon-fibers (especially Nylon 6) and resins. It is also used in brush bristles, textile stiffeners, film coatings, synthetic leather, plastics, plasticizers, paint vehicles, cross-linking agent for polyurethanes, and in the synthesis of lysine [1]. The most probable routes of human exposure to CA are dermal contact and inhalation which is mainly occurred in industry setting. Caprolactam is moderately toxic with oral LD50 of 1.2–1.6 g/kg and 4-h LC50 value of 8160 mg/m^3 in rat [2,3]. The potential health hazards associated with CA are irritation of respiratory tract, eye, skin and mucous membrane [4–7]. In rats with a single subcutaneous dose (0.5 g/kg) of CA, toxic effects including stupor, convulsion and death were observed [2]. The major urinary metabolite of CA was 4-hydroxycaprolactam (4-OHCA) or the corresponding free acid (16% of the dose) and a small amount as the non-hydroxylated acid, 6-aminocaproic acid (6-ANCA) in male Sprague-Dawley rats given 3% CA in the diet for 2–3 weeks [8].

Urine is a useful sample, particularly in the practice of biological monitoring, which may provide a useful indicator of exposure to toxic substances for several diseases. They are simple, non-invasive to collect, and available in relatively large quantities. In addition, urine samples contain relatively high concentrations of drugs and metabolites compared with other biological specimen, such as blood, hair and saliva. In recent years, in daily operation, many forensic and toxicology labs have been switching to liquid chromatography tandem mass spectrometry (LC–MS–MS) methods, which reduce the time and effort required in the tedious procedures of sample preparation and handling; improve detection limits; and faster analytical run times [9]. As far as we know, only high pressure liquid chromatography (HPLC) has been developed for the analysis of CA in biological samples in a previously published article [10].

In clinical setting, and particularly if an emergent intoxication is suspected, the determination of unusual substances is often requested in a very short of time. Therefore, to fit clinical need,

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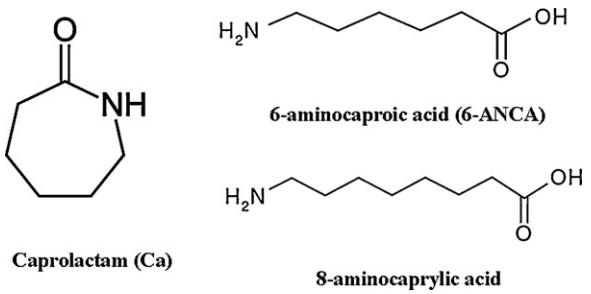

Fig. 1. The chemical structures of caprolactam, 6-aminocaproic acid and internal standard.

the turnaround time of such kind of test supposedly to be as short as possible (e.g. within 2 h). The present study describes the development and validation of a simple and rapid method for simultaneous determination of CA and 6-ANCA in urine sample, using hydrophilic interaction liquid chromatography (HILIC) coupled with electrospray ionization tandem mass spectrometry. The method was fully validated for the determination of CA and 6-ANCA from urine sample and applied in a case of caprolactam poisoning.

2. Experimental

2.1. Chemicals and reagents

All solvents and chemicals were of HPLC grade. Acetonitrile and ammonium acetate were purchased from Merck (Darmstadt, Germany). Formic acid was purchased from Sigma–Aldrich (St. Louis, MO, USA). Methanol was purchased from Mallinckrodt *Baker*, Inc. (Phillipsburg, NJ, USA). Drug standards of CA, 6-ANCA and 8-aminocaprylic acid were purchased from Sigma–Aldrich. The structures of CA, 6-ANCA and 8-aminocaprylic acid are shown in Fig. 1. The stock standard solutions of CA and 6-ANCA were prepared at 100 μg/mL with methanol and deionized water, respectively. A stock standard solution of 8-aminocaprylic acid (IS) at 100 μg/mL was also prepared in deionized water. The stock solutions were stable for 1 year at −20 °C. The stock standard solutions of CA and 6-ANCA were further diluted with acetonitrile/deionized water (9:1, v/v) to prepare the working standard solution containing (10 μg/mL) of CA and 6-ANCA. The stock solution of 8-aminocaprylic acid was further diluted with acetonitrile/deionized water (9:1, v/v) to prepare the working internal standard solution containing (10 ng/mL) of 8-aminocaprylic acid. The working solutions were stored at −4 °C until use.

2.2. Sample preparation procedure

The urine samples were diluted with acetonitrile/deionized water (9:1, v/v). Diluted samples (100 μL) were fortified with 50 μL (10 ng/mL) of internal standard working solution. Samples were briefly vortex-mixed for 10 s and centrifuged to remove large particles (5 min at 2000 rpm). 20 μL of analyte was transferred to an autosampler vial for LC–MS–MS.

2.3. LC–MS–MS analysis

2.3.1. Instrumentation

LC–MS–MS analyses were carried out with an Agilent 1200 series vacuum degasser, binary pump, well plate autosampler, thermostatted column compartment, paired with an Agilent 6430 Triple Quad LC–MS, operated in electrospray ionization (ESI) mode (Agilent Technologies, Inc. Folsom, CA, USA). All data were acquired and analyzed using Mass Hunter software, version B01.03.

2.3.2. Chromatographic conditions

Chromatographic separation was performed on a Phenomenex Luna HILIC column (100 mm × 2.0 mm I.D., 3 μm). Gradient elution with (A) 10 mM ammonium acetate in deionized water, 0.1% formic acid and (B) 10 mM ammonium acetate in 95% acetonitrile and 5% deionized water, 0.1% formic acid and at a flow rate of 0.3 mL/min was applied. The following gradient program was used: 0–2 min: 0% A, 100% B; 2–3 min: 0–30% A, 100–70% B; 3–5.5 min: 30–40% A, 70–60% B; 5.5–8 min: 40–50% A, 60–50% B. The HPLC column was re-equilibrated for 5 min, giving a total run of 13 min. The column temperature was maintained at 25 °C, and the autosampler was at room temperature. The valve was set to direct LC flow to the mass spectrometer from 0.5 to 8 min. The remaining time the LC eluent was diverted to waste.

2.3.3. MS detection

The Agilent 6430 Triple Quad LC–MS was used in the positive ESI multiple reaction monitoring (MRM) mode. The nitrogen drying gas temperature was 325 °C, and the flow was 10 L/min, nebulizer gas (nitrogen) 35 psi, and the capillary voltage was 3500 V. Dwell times were 50 ms.

2.4. Analytical method validation

2.4.1. Linearity

The method linearity for CA and 6-ANCA was investigated in the range from 62.5 to 1250 ng/mL (62.5, 125, 250, 500, 1000, 1250 ng/mL), and 31.25 to 1000 ng/mL (31.25, 62.5, 125, 250, 500, 1000 ng/mL), respectively. Matrix-matched calibrators were prepared by fortifying drug-free urine with the appropriate working solution. 50 μL of internal standard (10 ng/mL) was added and samples were analyzed following the complete procedure. Calibration curves were established with three replicates at each concentration. Concentrations of each calibrator were required to be within ±20% of target, when calculated against the full six-point curve.

2.4.2. The limit of detection (LOD) and the limit of quantitation (LOQ)

Sensitivity was evaluated by determination of the LOD and the LOQ. A series of decreasing concentrations of drug-fortified urine was analyzed to determine LOD and LOQ. The LOD was defined as the lowest concentration with acceptable chromatography, the presence of precursor and product ions with the ion ratios within ±20% of calibrator, and relative retention time within ±2% of retention time of calibrator. The LOQ was defined as the concentration where the measured value was within ±20% of the theoretical concentration, all qualifier ion ratios were within ±20% of the ion ratios based on the calibrator and relative retention time within ±2% of retention time of calibrator.

2.4.3. Accuracy and precision

Different concentrations of spiked urine (62.5, 400, and 800 ng/mL) for CA and (31.25, 400, and 800 ng/mL) 6-ANCA were analyzed ($n = 3$) in 1 day for the intra-day precision study. For the inter-day precision study, each concentration was analyzed on 6 different days. To determine the precision, the coefficients of variations (% CV) were calculated for replicate measurements. Accuracy (%) was expressed as the relative error from the expected value. It was calculated by the degree of agreement between the measured and nominal concentrations of the fortified samples.

2.4.4. Stability

Short-term stability was evaluated for urine fortified with the analytes of interest at 100, 400 and 800 ng/mL. Seven days

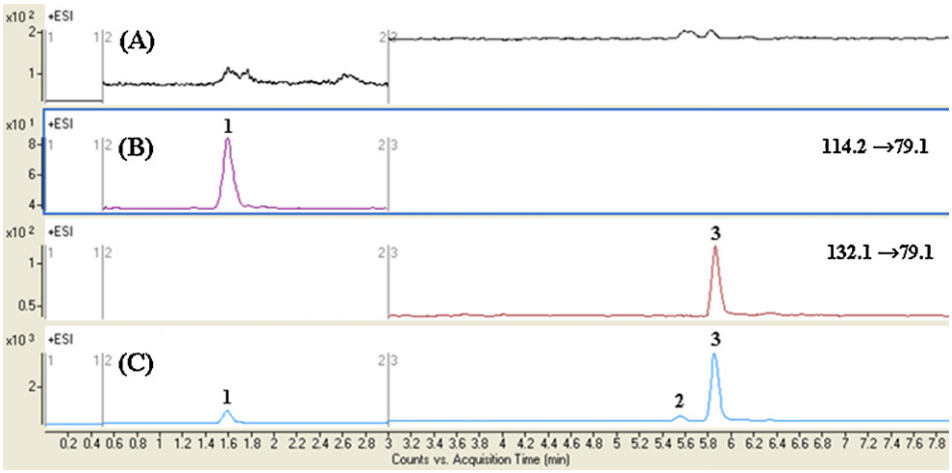

Fig. 2. (A) LC–MS–MS total ion chromatograms of a blank urine sample; (B) LC–MS–MS MRM chromatograms of blank urine spiked with 62.5 ng/mL of CA (top) and 31.25 ng/mL of 6-ANCA (bottom); and (C) LC–MS–MS total ion chromatograms of a urine sample of poisoning case containing 778,189 ng/mL CA and 347,338 ng/mL 6-ANCA. (1) Caprolactam; (2) internal standard; and (3) 6-aminocaproic acid.

stability was tested for samples stored at 4 °C. Each experiment was performed in duplicate.

2.4.5. Assessment of matrix effects

The assessment of matrix effect was based on the direct comparison method as described by Matuszewski et al. [11]. A set of samples containing the same amount of analytes in matrix-free solvent and blank urine specimens were used. By comparing the peak areas of the analytes standards in matrix free solvent (A), standards spiked into urine specimens after extraction (B).

$$\text{Matrix effect}\ (\%) = \frac{\text{Area ratio from}\ (A)}{\text{Area ratio from}\ (B)} \times 100$$

3. Results and discussion

3.1. Sample pretreatment

From the animal data, 4-OHCA was considered to be the major metabolite of CA. And theoretically, in addition to CA, we were supposedly to study the sequential change of 4-OHCA concentration in urine as well. However, the standard of 4-OHCA was not commercially available, therefore, instead of 4-OHCA, 6-ANCA, another metabolite, was chosen as a study item in this study.

Urine sample is high aqueous and has low protein content; therefore, it did not require extensive cleanup procedures prior to LC–MS–MS analysis. In addition, pre-concentration during sample preparation was not required based on the high sensitivity achieved by the LC–MS–MS method. For the reasons, direct injection of 20 μL of urine onto the LC–MS–MS system proved to be simple and efficient.

3.2. HPLC

The metabolite was more polar than parent compound, and consequently the high polarity of 6-ANCA is difficult to retain on analytical columns. A popular alternative for the separation of highly polar or hydrophilic compounds is HILIC, first introduced by Alpert in 1990, which is a pseudo-normal phase chromatographic technique [12]. It operates on the basis of hydrophilic interactions between the analytes and the stationary phase [13,14]. In the present study, the chromatographic separation of CA and 6-ANCA was promptly completed using HILIC column with acetonitrile/deionized water gradient elution. The LC–MS–MS chromatograms of blank urine, urine sample at LOQ (62.5 ng/ml for CA and 31.25 ng/mL for 6-ANCA) and a poisoning case are shown in Fig. 2(A–C). The retention times of CA, 6-ANCA and the IS were 1.58, 5.85 and 5.55 min, respectively. In addition, the use of formic acid and ammonium acetate in mobile phase provided a simple procedure to effectively enhance the resolution of LC separation and the sensitivity of MS–MS detection for CA and 6-ANCA. Furthermore, reducing the amount of matrix reaching the mass spectrometer, a divert valve directed the LC eluent to waste after separation of the target analytes. This proved to be an easy and practical solution to ensure cleanliness of the ion source and reduce maintenance of the mass spectrometer.

3.3. MS–MS

The MS–MS method was optimized to obtain the highest response using the MRM pairs comprising the precursor and product ion(s). Precursor and product ion(s) for each analyte of interest were determined by direct infusion of single analyte solutions (10 μg/mL in mobile phase). Unique precursor–product ion(s) combinations were selected to develop a quantitative LC–MS–MS method based on MRM. After optimization, the protonated precursor molecular ions $(M+H)^+$ were chosen to be monitored. The MS–MS spectrums were shown in Fig. 3. The primary mass transition of CA was mass/charge ratio (m/z) 114.2–79.1 (collision energy, 17 V; fragmentor energy, 126 V). The secondary transition of CA was m/z 114.2–55.1 (collision energy 25 V; fragmentor energy 126 V). The primary transition and secondary transition of 6-ANCA were m/z 132.1–79.1 and m/z 132.1–69.1, respectively (collision energy 13 V; fragmentor energy 77 V). The primary mass transition of IS was mass/charge ratio (m/z) 160.2–142.1 (collision energy, 5 V; fragmentor energy, 82 V). The secondary transition of IS was m/z 160.2–79.1 (collision energy 13 V; fragmentor energy 82 V). In addition, the chromatographic run was divided into two segments, each containing a set of optimized MS parameters for the compounds of interest eluting within a given time period to insure maximum sensitivity of the MS analysis.

3.4. Evaluation of validation data

The method was validated by verifying method linearity, LOD, LOQ, intra- and inter-day precision, and accuracy. Table 1 presents the parameters for the quantitative validation of the methods, including linearity covering the concentration range of

Table 1
Validation data.

Compound	Calibration range (ng/mL)	Linearity[a] (r^2)	LOD[b] (ng/mL)	LOQ[c] (ng/mL)
CA	62.5–1250	0.998	62.5	62.5
6-ANCA	31.25–1000	0.996	15.6	31.25

[a] Linearity is described by the correlation coefficient of the calibration curve.
[b] LOD was determined as the lowest concentration with acceptable chromatography, the presence of precursor and product ions with an ion ratio within ±20% of calibrator, and relative retention time within ±2% of average retention time of calibrator.
[c] LOQ was determined as the concentration at which all acceptance criteria are met and the quantitative value is within ±20% of the target concentration.

Table 2
Intra-day and inter-day precision and accuracy.

Compound	Nominal concentration (ng/mL)	Intra-day ($n=3$)		Inter-day ($n=6$)	
		Precision (% CV)[a]	Accuracy (bias)[b]	Precision (% CV)[a]	Accuracy (bias)[b]
CA	62.5	8.7	−5.3	9.9	−6.1
	400	3.2	3.5	5.6	2.7
	800	2.3	1.3	6.3	−1.0
6-ANCA	31.25	7.2	−2.6	8.1	6.6
	400	2.2	−0.5	2.5	−5.7
	800	1.6	1.0	4.9	−3.9

[a] The coefficient of variance (% CV): SD/mean × 100%.
[b] Calculated as [(mean calculated concentration − nominal concentration)/nominal concentration] × 100.

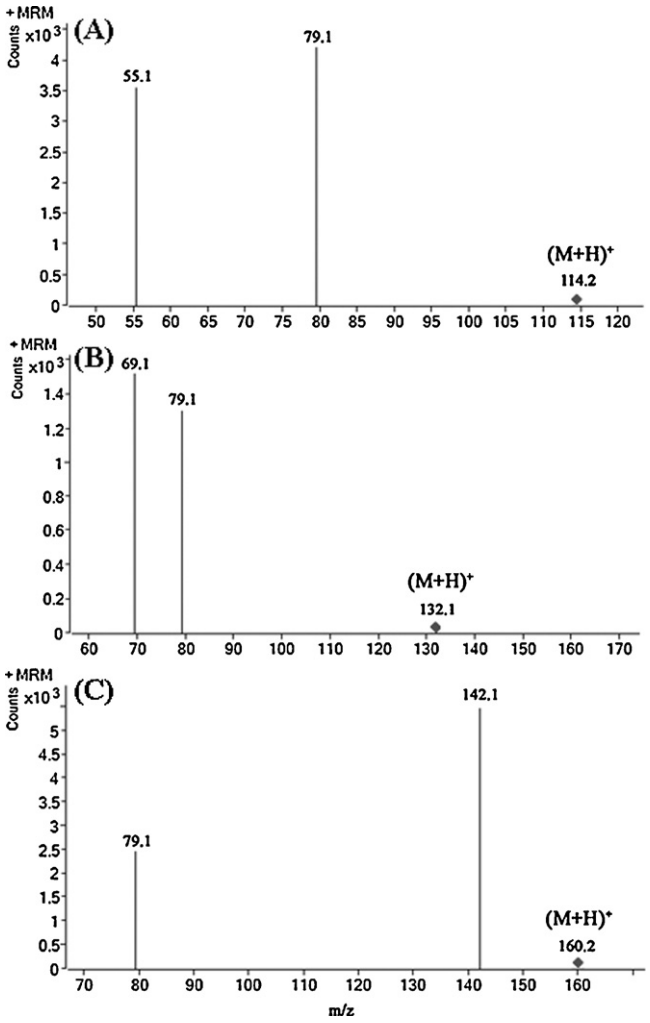

Fig. 3. LC–MS–MS mass spectra of (A) caprolactam; (B) 6-aminocaproic acid, and (C) internal standard from a spiked urine sample.

62.5–1250 ng/mL for CA and 31.25–1000 ng/mL for 6-ANCA. The correlation coefficient of calibration curves were ≥0.995 for all analytes, indicating significant linear regression. The sensitivity of the method was evaluated by determining the LOD and the LOQ for each analyte. The LOD and LOQ values obtained were 62.5 ng/mL and 62.5 ng/mL for CA, 15.6 ng/mL and 31.25 ng/mL for 6-ANCA. The analytical precision and accuracy were evaluated at three concentrations (low, middle, high), covering the linear dynamic range of each analyte. The results of the intra-day and inter-day precision and accuracy experiments are given in Table 2. The intra-day precision and accuracy of all three concentrations ranged from 1.6% to 8.7% and from 5.3% to 3.5%, respectively; and the inter-day precision and accuracy values ranged from 2.5% to 9.9% and from 6.1% to 6.6%, respectively. Seven days stability of the compounds was evaluated after stored at 4 °C. No significant variability in CA concentration was observed in 7 days stability tests. However, there was observable degradation of 6-ANCA on the third day. Therefore, we suggest that the sample should be analyzed within 3 days.

Evaluating matrix effects is of the utmost importance when developing a quantitative LC–MS–MS method. The ionization efficiency of the analyte might be influenced when matrix and analytes enter the ion source at the same time. It was found that matrix effect could result in poor analytical accuracy and reproducibility. Therefore, it is important to eliminate matrix effect to obtain reliable analytical results. Several approaches have been used to minimize the matrix effect. These include extensive sample cleanup procedure, improvement of the LC separation to avoid the co-elution of the analyte(s) with matrix, or serial dilution of the final extract, such that fewer matrix components will be injected into the analytical system. After our evaluation, it is helpful to compensate for the matrix effect by diluting the final extract.

The LC–MS–MS method fulfilled our analytical standard criteria. Shorter specimens processing time, and the detection limits, inter-day and intra-day reproducibility were all acceptable. No matrix effect was observed despite the lack of sample pre-treatment.

3.5. Analysis of clinical samples

A 30 year-old female was referred to our emergent department with initial presentations of convulsion and conscious change. The patient was well in the past and had been working in the plastic recycling industry for 3 months. She experienced vomiting and

Table 3
The concentration of CA and 6-ANCA in urine specimens collected from the poisoned case.

Specimen	Sampling hours after seizure attack (h)	Concentration (ng/mL)	
		CA	6-ANCA
1	17	778,189	347,338
2	21	598,925	419,215
3	25	525,528	503,257
4	29	449,272	723,062
5	32.5	435,189	695,009

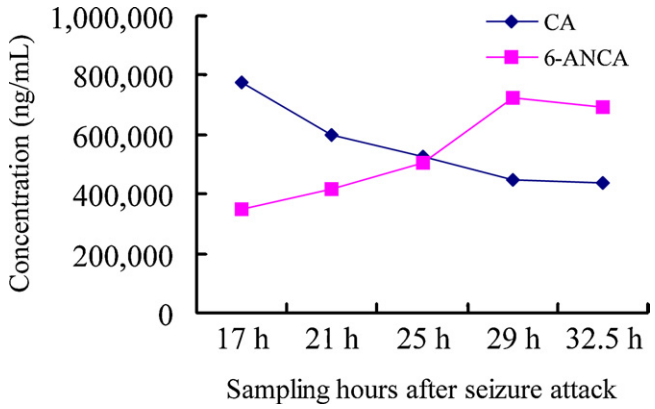

Fig. 4. The urinary concentration–time profiles of CA and 6-ANCA.

lethargy while finishing her daily works. Seizure attack was noted on the way to the hospital. Physical examination revealed erythematous change and pigmentation of bilateral elbows and thighs. Laboratory tests showed leukocytosis, hypokalemia and metabolic acidosis. Electroencephalogram revealed abnormal cerebral activity in bilateral temporal area. History also revealed that she has taken some other medicines for stomach pain and common cold for 1 day. Clinically, her presentation was with caprolactam (the active ingredient of plastic material) poisoning. We were wondering if the poisoning was resulted from the interaction of the chemical (caprolactam) exposure and the medicine (cimetidine) taken by herself.

The developed method was applied to determine the urinary concentration of CA and 6-ANCA for poisoned case of caprolactam. Table 3 shows the results of CA and 6-ANCA analysis from urinary samples collected at five different time points. The urinary concentration of CA and 6-ANCA was found to range from 435,189 to 778,189 ng/mL and 347,338 to 723,062 ng/mL, respectively. The maximum CA concentration was observed at 17 h after seizure attack. Thereafter, urinary CA levels decreased, and in the meanwhile, 6-ANCA levels increased steadily over time (Fig. 4). Since, urinary concentration change of CA and 6-ANCA for the first 17 h was not available, it is difficult to estimate the kinetic as well as the metabolic pattern of CA in this case. However, these results shows that the analytical method describes here, holds a valuable potential in understanding the kinetic change of caprolactam in clinical poisoning.

4. Conclusions

In the present study, the LC–MS–MS method using a HILIC column was developed for the simultaneous determination of CA and 6-ANCA in urine, without sample preparation. Sensitive and precise quantification were observed after internal standard addition and direct injection of urine onto the LC–MS–MS system. The method fulfilled our analytical validation criteria, and the applicability of the LC–MS–MS method was demonstrated by the analysis of poisoned urine samples. CA poisoning can be confirmed by detecting parent compound in human body. The metabolites found in human urine can testify the CA absorbed, and provide chemical information for identification of CA poisonings. The analytical method is potentially to be useful for pharmacokinetic and metabolic studies of caprolactam. The application of the method in one real case demonstrated that our method for the quantitative determination of CA and 6-ANCA in urine is ready to be used in the analysis of clinical and toxicological samples.

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