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ORIGINAL ARTICLE

Development and validation of an analytical method for determination of bongkrekic acid in biofluids for toxin monitoring

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Abstract

Bongkrekic acid (BKA) poisoning is a severe foodborne illness with a high mortality rate. This study aimed to identify BKA in postmortem tissues from poisoning victims in Taiwan and to develop a reliable analytical method for detecting BKA in biofluids to aid clinical diagnosis and treatment. BKA was identified in postmortem samples using high-resolution mass spectrometry (HRMS) and confirmed with an independent ultrahigh-performance liquid chromatography coupled with tandem mass spectrometry (UHPLC-MS/MS) method. To meet clinical needs, a UHPLC-MS/MS method was developed and validated for BKA detection in plasma and urine. Method optimization included adjustments to ion source conditions for multiple reaction monitoring (MRM) transitions and avoidance of glass vials due to BKA adsorption onto free silanol groups. Chromatographic separation was achieved using a 50-mm Hypersil Gold C18 column within a 6-min run time. The validated UHPLC-MS/MS method successfully detected BKA in biofluids, enabling its application in identifying victims of foodborne poisoning. The method demonstrated high accuracy and efficiency, facilitating timely diagnosis and aiding in treatment strategies for critically ill patients. The developed UHPLC-MS/MS method provides a reliable approach for detecting BKA in clinical and forensic settings. Its implementation enhances diagnostic capabilities, improves patient outcomes, and supports monitoring of toxin elimination in cases of BKA poisoning.

Keywords: Biofluids, Bongkrekic acid, Clinical analysis, Diagnosis, LC-MS/MS

1. Introduction

Foodborne poisoning presents significant public health risks [1], presenting challenges in prevention due to diverse microbial hazards. According to the World Health Organization (WHO), nearly 1 in 10 people suffered from a foodborne illness in 2010, resulting in 420,000 deaths [2]. The spread of foodborne pathogens remains a persistent concern, especially with the rise of globalization [3] and the abruption of changes in climate [4]. For example, bongkrekic acid (BKA), produced by *Burkholderia gladioli pathovar cocovenenans*, is a strong and lethal mitochondrial toxin and has been associated with multiple foodborne poisoning outbreaks in China and Indonesia [5]. It was not reported in other

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regions until an outbreak occurred in Mozambique in 2015 [6,7]. Additionally, the first death from BKA in North America was reported in 2024 [8]. That same year, an outbreak of food contamination in Taiwan resulted in multiple casualties, and BKA, which had not been previously detected in the region, was identified [9]. BKA originates from fermented or spoiled corn or coconut products [10]; however, it can also be derived from nonfermented foods and unspoiled foods [11]. More threatening, the BKA-contaminated foods can have a normal appearance and taste since BKA is odorless and tasteless, making it more challenging to prevent BKA poisoning [5].

BKA poisoning can rapidly progress from liver and kidney damage to systemic multiple organ failure, with a high case fatality rate of 29.5% in an epidemiological study from 2010 to 2020 in China [12]. A previous study revealed that 1–1.5 mg of BKA can be fatal to humans [13], as BKA specifically binds to adenine nucleotide translocase (ANT) on the mitochondrial inner membrane and blocks the exchange of adenosine triphosphate (ATP), resulting in severe cell damage [5]. Symptoms of BKA poisoning, including vomiting, diarrhea, abdominal pain, weakness, vertigo, nausea, palpitation, and chest pain, have been reported [5,6,12]. However, these clinical signs are not specific to the intoxication of BKA. Thus, reaching a differential diagnosis of BKA poisoning relies on the detection of BKA in the victims. Currently, there is no antidote for BKA poisoning. Supportive therapies such as intermittent hemodialysis (HD), continuous renal replacement therapy (CRRT), and plasma exchange (PE) have been reported and utilized in treating patients in critical condition [14]. Since BKA is likely to have a very large volume of distribution (Vd) due to its high lipophilicity, eliminating the toxin efficiently is challenging. A previous study evaluated the BKA remaining in circulation between therapies [15], and it is crucial to achieve this goal with validated analytical methods.

Currently, the analytical methods used to determine the presence of BKA are focused mainly on food materials. Ultrahigh-performance liquid chromatography coupled with tandem mass spectrometry (UHPLC-MS/MS), along with various extraction methods, is most commonly employed [16–18]. Other methods, such as UV–Vis with gold-nanoparticles [19] and immunoassays [20–22], have been used to study BKA levels in various food matrices. Analysis of BKA in biofluids is relatively scarce in the literature. Previous studies have employed UHPLC-MS/MS to analyze the plasma concentrations of BKA [10,15] via the direct protein precipitation method. However, these methods lack appropriate internal standards that might suffer from matrix interferences. Thus, more complex methods, such as the dispersive liquid–liquid microextraction method, have been utilized to perform sample clean-up to minimize matrix interference in clinical plasma samples [23]. Nowadays, synthetic isotope-labeled standards are commercially available, and the correction of matrix effects has become achievable.

In this study, we first described the process for identifying BKA in postmortem tissues and developed a rapid, accurate method to measure BKA levels in biofluids, aiding in clinical differential diagnosis and facilitating timely monitoring of toxin elimination.

2. Material and method

2.1. Chemicals and reagents

BKA, MS-grade methanol, acetonitrile, and formic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Isotope-labeled BKA ($^{13}C_{28}$-BKA) was acquired from MedChemExpress (Monmouth Junction, NJ, USA).

2.2. Samples and sample preparation

Forensic specimens, including gastric contents and whole blood, were collected from two food-borne poisoning victims during autopsy and were provided by the Institute of Forensic Medicine, Ministry of Justice, Taiwan. Clinical biofluids, including plasma and urine, were collected from patients who were suspected to be victims of food-borne poisoning incidents. These samples were provided by the Taiwan Centers for Disease Control, Ministry of Health and Welfare, Taiwan. This study was approved by the Institutional Review Board of MacKay Memorial Hospital (IRB: 24MMHIS179e), Taipei Medical University Hospital (TMU-JIRB No: N202405072) and Shin Kong Wu Ho-Su Memorial Hospital (IRB No: 20240814R). For forensic specimen analysis, a total of 50 μL of gastric contents and whole blood was aliquoted into a 1.5-mL microtube, followed by the addition of 200 μL of MeOH for protein precipitation. The supernatants were collected after centrifugation at 10,000 rcf for 15 min prior to LC-MS analysis. For urine BKA analysis, 20 μL of urine was mixed well with 20 μL of IS solution (50 ng/mL) and 160 μL of 50% MeOH. Following centrifugation at 10,000 rcf for 15 min, clear supernatants were transferred into PP auto-sampler vials. For each plasma sample, 50 μL of

plasma was mixed well with 50 μL of IS solution (50 ng/mL), and 150 μL of ACN was added for protein precipitation. An aliquot of 100 μL of the supernatant was removed and diluted with 100 μL of DI water prior to LC-MS analysis.

2.3. LC-MS/MS

For identification of BKA in forensic specimens, a UPLC H-class coupled to high-resolution QTOF (Synapt XS, Waters, Milford, MA, USA) with data-dependent acquisition (fast DDA) mode was employed. The top 10 signals from the survey scan were selected for MS/MS with ramping collision energy in the centroid mode. The mass range was set from m/z 50–1200 in negative mode. The m/z 554.2615 in the negative mode generated from leucine enkephalin was utilized for lock mass. The MS source parameters were set as 2.5 kV, 120 °C, 40 V, 300 °C, 600 L/Hr, and 6.5 bar for the capillary voltage, source temperature, sampling cone, desolvation temperature, desolvation gas flow and nebulizer gas flow, respectively. A 150 mm Hypersil Gold C18 column (150 × 2.1 mm, 1.8 μm, Thermo Fisher Scientific, Waltham, MA, USA) was used to separate the BKA. The mobile phases were composed of 0.1% formic acid in MS-grade water (A) and 0.1% formic acid in acetonitrile (B). The gradient conditions were set as follows: 0–1 min, 20% B; 1–8 mins, 20–95% B; 8–12 mins, 95% B. A 3-min equilibrium was employed prior to the next run. A 5 μL aliquot of each sample was injected onto a column for LC–MS/MS analysis. For the quantification of BKA in biofluids, a UHPLC 1290 (Agilent Technology, Santa Clara, CA, USA) coupled to a triple quadrupole 6500 (AB Sciex, Framingham, MA, USA) in multiple reaction monitoring (MRM) mode was used to perform LC-MS/MS analysis. A biphenyl column (2.7 μm, 2.1 × 100 mm) from Phenomenex (Torrance, CA, USA) and a 50 mm Hypersil Gold C18 column (50 × 2.1 mm, 1.8 μm, Thermo Fisher Scientific, Waltham, MA, USA) were used to test the analytical performance. The mobile phases consisted of 0.1% formic acid in water (A) and 0.1% formic acid in 100% methanol (B). The gradient conditions were set as follows: 0–0.5 mins, 10% B; 0.5–2 mins, 10–50% B; 2–3 mins, 50–98% B; and 3–4 mins, 98% B. A 2-min equilibrium was employed prior to the next run. The autosampler temperature was set at 10 °C. The injection volume was 10 μL. The mass spectrometer was operated in negative ionization mode with the ion spray voltage set to 5500 V, nebulizer (gas 1) pressure set to 50 psi, drying gas (gas 2) pressure set to 60 psi, and gas temperature set to 400 °C. The curtain gas pressure was 30 psi. The MRM method contained at least two transitions for the analyte and ISTD.

2.4. Method validation

Method validation was executed following the FDA bioanalytical guidelines [24]. The calibrators and quality control samples were prepared in blank heparin plasma and blank urine. Calibrator samples were prepared at 2.5, 5, 10, 20, 50, 100, 200 and 500 ng/mL. The quality control samples were prepared at concentrations of 5, 50 and 200 ng/mL in blank urine and plasma. Calibration curves were established by using the ratio of the analyte peak area against the IS area versus the nominal concentration of standards with weighted linear regression (1/x). The acceptance criterion for a calibration curve was a coefficient of determination R^2 of 0.990 or better. The limits of detection (LOD) and lower limits of quantification (LLOQ) were evaluated by analyzing serially diluted standards in plasma and urine specimens, repeated 3 times at varying concentrations in the range of 0.5 ng/mL to 50 ng/mL. The LLOQs were defined as the concentration at which the percent coefficient of variation (% CV) was 20%. The accuracy acceptance criterion for LLOQ determination was ± 20%. The intraday (within-day) precision was calculated based on five repeated analyses of independent control plasma samples spiked with the tested compounds.

2.5. Data analysis

For BKA identification, high-resolution MS results were processed with MassLynx 4.2 (Waters, Milford, MA, USA). The quantification of BKA using the acquired MRM results were processed by Skyline [25]. Statistical analysis was performed by Microsoft Excel and R language.

3. Results

3.1. Identification of BKA in forensic tissues and blood from the first two victims

We received forensic specimens from two patients suspected to have died from food poisoning at the end of March 2024. Based on the clinical findings, which revealed severe liver damage after consuming wet rice noodles at the same restaurant—similar to cases described in previous literature [26]—the clinicians speculated that BKA intoxication might be the cause, despite no prior reports of BKA cases in Taiwan. To test this hypothesis, we used two

independent LC-MS/MS instruments, UPLC-QTOF and UHPLC-QqQ, to determine the presence of BKA in these forensic specimens. We first analyzed the gastric contents and postmortem blood specimens from case #1 (male, 39-yr) and the gastric contents from case #2 (male, 66-yr) using a high-resolution mass spectrometer (HRMS) coupled with UPLC and a 15-cm C18 column (150 × 2.1 mm, 1.8 μm). As shown in Fig. 1A, BKA signals (extracted ion chromatograms of m/z 485.2539, $[M-H]^-$ ion of BKA in negative mode, within 10 ppm error) were observed in the forensic specimens and standard solution at 7.20 min but not in the control blood sample or solvent blank. The high-resolution MS spectra at 7.20 min, as illustrated in Fig. 1B, demonstrated that accurate masses (m/z 485.2546) matched the theoretical mass of BKA (m/z 485.2539) with a 1.4 ppm error. In addition, we acquired MS/MS spectra at 7.20 min with a precursor of m/z 485 and a collision energy of 20 V, as shown in Fig. 1C. The MS/MS spectrum of case #1 blood was identical to that of the BKA standard. Furthermore, we employed another independent MS system with various LC columns and mobile phases to confirm these results. A 10-cm biphenyl column with methanol/water-based mobile phases, coupled to a triple quadrupole MS in MRM mode, was utilized to analyze BKA. As shown in Supplemental Fig. S1 (https://doi.org/10.38212/2224-6614.3549), the retention times and ion ratios of the BKA MRM transitions obtained from the forensic specimens were identical to those of the reference standard. Considering the match of retention time, MS/MS spectra and accurate mass between the forensic specimens and the BKA standard, we were more confident in the conclusion that the cause of the foodborne poisoning was BKA contamination of the food materials in the restaurant.

3.2. Method development for determination of BKA in biofluid specimens

Following the announcement that the BKA was identified as the cause of the food poisoning incident, it is highly important to recognize the source of the BKA and clarify how many victims were involved in this incident. Since the symptoms are not very specific, it would be critical to detect the presence of BKA in plasma and urine specimen to determine if the patient was poisoned with BKA. In addition, for patients in critical condition, it is also essential to monitor the toxin concentrations to further adjust treatment strategies. Thus, there is an urgent need to develop an effective method to analyze BKA levels in biofluids.

We first optimized the MS parameters to obtain optimal MRM transitions. The BKA standard solution was prepared in MeOH at a concentration of

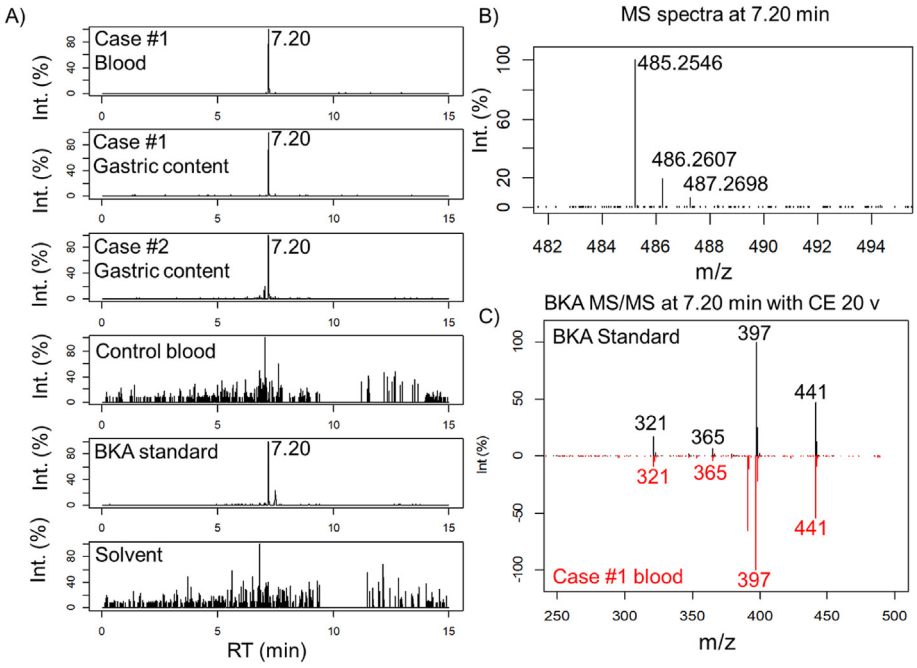

*Fig. 1. **Identification of the presence of BKA in forensic specimens with HRMS.** A) Extracted ion chromatograms of m/z 485.1239 in case #1 blood, gastric content, case #2 gastric content, control blood, BKA standard (100 ng/mL), and solvent blank, respectively. B) MS spectra at 7.20 min of the case #1 blood sample. C) MS/MS spectra at 7.20 min with 20 V collision energy (CE) in the BKA standard (top) and case # blood (bottom).*

100 ng/mL and infused into the MS with ramping voltages and collision energies. The fragment ions at m/z 441 $[M\text{-}H\text{-}CO_2]^-$ and m/z 394 $[M\text{-}H\text{-}(CO_2)_2]^-$ were the dominant product ions and were employed as quantification and qualification ions, respectively. The finalized MRM transitions for BKA and its stable isotope standard are listed in Table 1. In addition, to increase the detection sensitivity, we examined the effects of the ion source parameters on the peak signals. As shown in Fig. 2, the temperature of the ion source had a significant impact on the ionization efficiency. Compared with a source temperature of 600 °C, a temperature of 400 °C yielded an approximately sevenfold increase in the peak response.

Moreover, we tested different analytical columns, including a biphenyl column (BP, 100 × 2.1 mm, 2.6 μm) and a Hypersil Gold C18 column (HG, 50 × 2.1 mm, 1.9 μm). Both columns exhibited good peak shapes and enough capacity (K), as shown in Supplemental Fig. S-2A (https://doi.org/10.38212/2224-6614.3549). Matrix effects were assessed by calculating the ratio of the signal intensity of post-spiked BKA in blood samples to that of a neat 50 ng/mL BKA standard solution. This evaluation was used to determine the extent of blood matrix interference on BKA signal detection when using the two tested columns. Compared with the HG column, the tested BP showed more severe matrix effects (as shown in Supplemental Fig. S-2B (https://doi.org/10.38212/2224-6614.3549)), suggesting that strong interferences in blood specimens were not able to be separated from BKA with the BP column. Although a heavy-labeled stable isotope standard was employed to correct the matrix effect, it would be better to have fewer matrix effects. Thus, the HG C18 column was chosen as the analytical column. Various mobile phases, including acetonitrile and methanol, were also tested, and there was no significant difference in chromatographic performance. Considering its environmentally friendly and analytical cost, methanol was selected as the mobile phase.

In addition, glass vials and polypropylene (PP) tubes are frequently used containers to store stock solutions in analytical chemistry. Surprisingly, we found that the peak area of the BKA standard placed in glass vials was much smaller than that in the PP tube. This could be due to the free silanol on the glass wall interacting with the carboxyl group of BKA in the solution, which is similar to the matrix effects in the GC liner [27,28] as illustrated in Fig. 3A. Thus, to verify this hypothesis, we adopted the concept of using analyte protectants to prevent the effects of the GC matrix. We rinsed glass vials with 1 mL of human plasma to deactivate the active sides of the glass vials. One milliliter of 10 ng/mL BKA solution was aliquoted into various containers, including 1.5-mL PP microtubes, brown glass vials (BG), clear glass vials (CG), and plasma-rinsed BGs. As shown in Fig. 3B, the peak area of BKA prepared in the PP tube exhibited the greatest response, whereas glass vials from various venders presented only an approximately 40% response compared with that of PP. Glass vials deactivated with plasma rinsing presented a slight reverse response (BG + AP). However, recovery with analyte protectants did not reach the optimal conditions. Thus, it would be better to prepare a working solution of BKA in a PP tube.

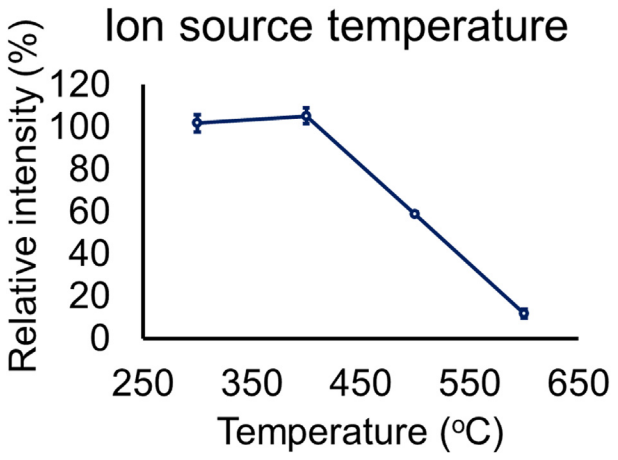

Fig. 2. Effect of ion source temperature on BKA ionization efficiency. (Normalized to the temperature at 300 °C, n = 3).

3.3. Method validation

The selectivity of the analytical method was examined, and no significant interfering peaks were

Table 1. MRM transitions for BKA and BKA-$^{13}C_{28}$.

Compound	Precursor (m/z)	Product (m/z)	Dwell (ms)	DP (v)	EP (v)	CE (v)	CXP (v)
BKA	485	441	100	−50	−10	−17	−25
BKA	485	397	100	−50	−10	−25	−25
BKA-$^{13}C_{28}$	513	469	100	−50	−10	−17	−25
BKA-$^{13}C_{28}$	513	425	100	−50	−10	−25	−25

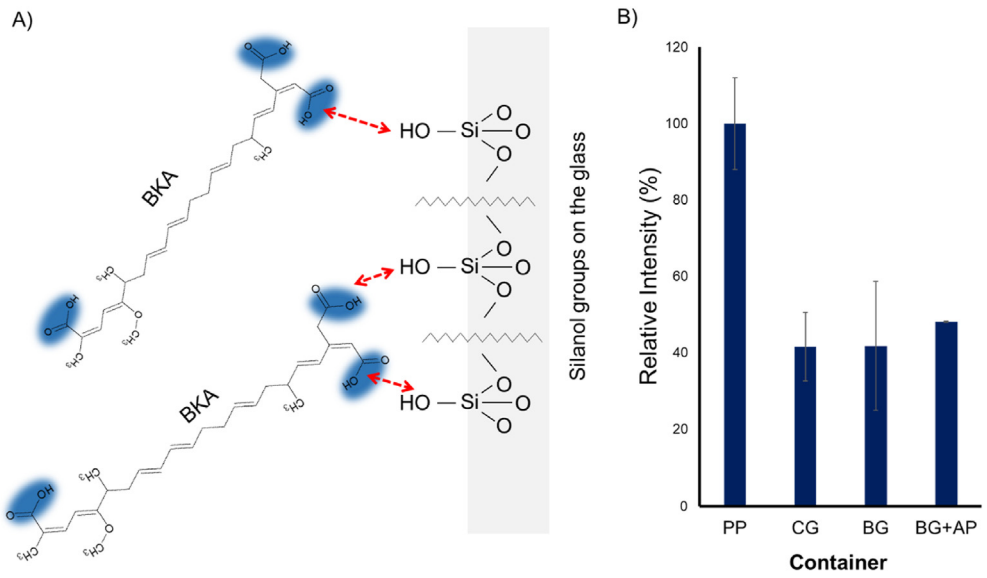

Fig. 3. Effects of the glass container on the adsorption of BKA. A) Possible mechanism of adsorption between free silanol on the glass wall and the carboxylic groups of BKA. B) Relative peak of 10 ng/mL BKA standard solution in MeOH prepared in PP, clear glass vials (CG), brown glass vials (BG) and BG with plasma pre-rinsing (Normalized to the PP group, n = 3).

found in the blank plasma and urine samples from the six healthy individuals.

The validation parameters are listed in Table 2. The calibration curves for BKA in plasma and urine were linear in the range of the tested concentrations from 2.5 to 500 ng/mL, with coefficients of determination (R^2) $\geq$ 0.9994. The LOD and the LLOQ were 1 and 2.5 ng/mL for both the plasma and urine matrices, respectively. Acceptable intra- and inter-day relative standard deviation (RSD) values ranging from 0.82 to 3.49% were obtained for BKA at the LQC, MQC and HQC. The accuracies ranged from 91.23 to 103.7%. These results suggest that the method is accurate and reliable.

3.4. Method application

We used the developed method to investigate foodborne poisoning incidents, aiming to identify victims and determine the extent of toxin elimination in severe cases.

Two weeks after the incident, 36 plasma and 22 urine samples from 36 individuals identified as potential victims were analyzed using the developed method, as shown in Fig. 4. BKA was detected in the plasma of 30 individuals, confirming that they were BKA poisoning victims (Fig. 4A). Six individuals had no detectable BKA in their plasma samples. Further investigations revealed that these six individuals were not patrons of the restaurant. Compared with plasma BKA concentrations, urine BKA concentrations were less conclusive, as BKA was undetectable in the urine of most patients with mild symptoms (Fig. 4B). Thus, plasma BKA could be a better diagnostic tool for detecting BKA poisoning.

Later, the authorities confirmed that all individuals who tested positive and had dined at the restaurant and consumed wet rice noodle products experienced severe food poisoning symptoms, including diarrhea and vomiting. However, how the food products became contaminated remains unclear and is still under investigation.

4. Discussion

Foodborne poisoning constitutes a significant public health concern. In this study, we were the first to identify and confirm the presence of BKA in postmortem tissues from two deceased victims of a foodborne poisoning incident through high-resolution mass spectrometry (HRMS) and an independent tandem mass spectrometry (MS) approach.

This represents the first documented case of BKA poisoning in Taiwan. A comprehensive understanding of the toxicological mechanisms and the ability to accurately identify victims necessitates the development of a robust analytical method for the quantification of BKA in biological fluids. Here, we present a rapid and sensitive method based on simple protein precipitation and matrix-matched calibration with the use of isotope-coded standards, enabling the precise monitoring of BKA in plasma and urine.

We have carefully optimized the analytical method. The ion source temperature and the use of glass containers can significantly impact the

Table 2. Method validations for analyzing BKA in plasma and urine.

	Range (ng/mL)	LOQ (ng/mL)	LOD (ng/mL)	Linearity	R^2	Intraday precisions (RSD, %)			Interday precisions (RSD, %)			Accuracy (Recovery, %)		
						LQC (5)	MQC (50)	HQC (200)	LQC (5)	MQC (50)	HQC (200)	LQC (5)	MQC (50)	HQC (200)
Plasma	2.5–500	2.5	1	$y = 0.0264x - 0.1067$	0.9994	2.78	3.77	3.39	1.8	0.82	1.15	103.70	91.23	103.51
Urine	2.5–500	2.5	1	$y = 0.0241x - 0.0838$	0.9998	2.79	3.11	1.8	3.49	2.07	2.24	99.52	92.37	102.1

detection of BKA. Our findings indicate that high ion source temperatures may suppress BKA ion generation, a phenomenon not previously reported. Additionally, we observed that BKA readily adsorbs onto glass surfaces, leading to a substantial underestimation of its concentration. This study is the first to report these effects, providing valuable insights for improving the accuracy of BKA quantification.

Moreover, while numerous studies have focused on determining BKA levels in food matrices as reviewed by previous study [29], only a few have examined its concentrations in human patients [10,15], resulting in limited understanding of the toxin's distribution and behavior in the human body. This highlights the urgent need for clinical toxicologists to monitor BKA levels *in vivo* in order to elucidate its mechanism of action. We reviewed several studies that monitored BKA concentrations in patients, as summarized in Supplemental Table S-1 (https://doi.org/10.38212/2224-6614.3549). Compared to these methods, our approach achieved comparable limits of detection (LOD, 0.5–3.7 ng/mL), limits of quantification (LOQ, 1–10 ng/mL), and analytical ranges in human plasma. Notably, matrix-induced ion suppression can reach up to 40% [10], consistent with our findings (Supplemental Fig. S-2B (https://doi.org/10.38212/2224-6614.3549)), underscoring the need to correct for matrix effects from complex biofluids to ensure accurate quantification. To our knowledge, this study is the first to utilize 13C isotope-coded BKA ($^{13}C_{28}$-BKA) as a stable isotope internal standard to effectively compensate for matrix effects in biofluid analysis.

Several patients developed severe multiple organ failure and were admitted to the intensive care unit (ICU). Currently, there is no antidote for BKA poisoning. Supportive therapies, including HD, CRRT and PE, have been utilized to treat patients in critical condition. In this context, evaluating toxin elimination is crucial for assessing the effectiveness of these supportive therapies, which relies on accurate quantification of BKA in biofluids. Therefore, we monitored BKA concentrations in the patients' biofluids using the developed method and provided the results to their medical teams to adjust treatment strategies. During the hospitalization period, we analyzed 209 plasma samples and 38 urine samples from these four patients. The lower number of urine specimens was due to severe renal dysfunction, which restricted daily urine collection. Unfortunately, these four patients did not survive, with survival durations of 35, 44, 73, and 81 days, respectively. However, timely plasma BKA concentration measurements are valuable for improving clinical treatment methods. We observed that the

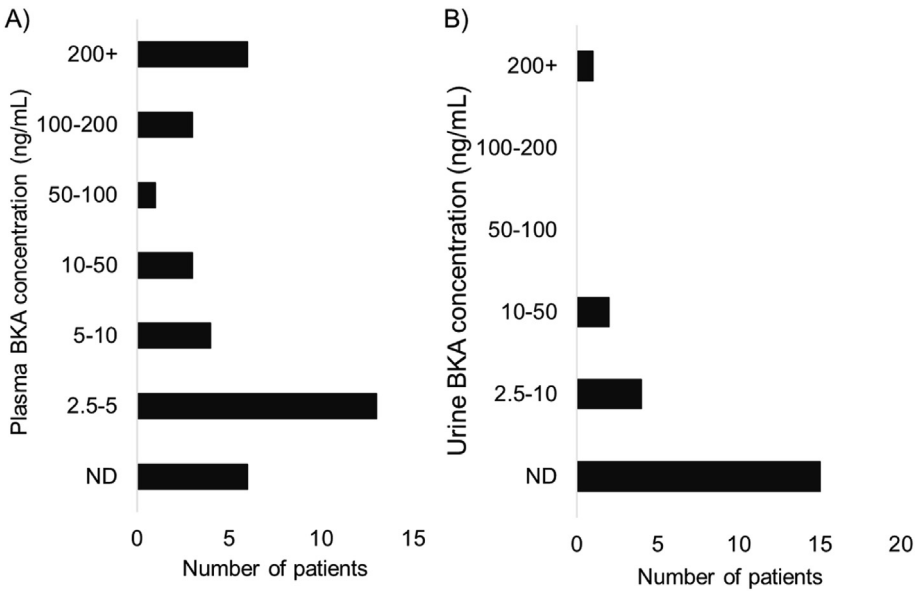

Fig. 4. Frequency distributions of BKA concentrations in the plasma (A) and urine (B) of suspected poisoned patients.

initial plasma concentrations of BKA, which were above 300 ng/mL in the patients, decreased to less than 20 ng/mL within 30 days. Taken together, the developed method is applicable for providing a differential diagnosis and monitoring the elimination of BKA in biofluids.

The method was successfully validated and applied to identify victims of foodborne poisoning incidents and to assist in assessing treatment strategies for critically ill patients. This method facilitates timely clinical diagnosis, enhances patient outcomes, and supports the efficient monitoring of toxin elimination in cases of BKA poisoning.

CRediT author statement

Ying-Tzu Shieh—Conceptualization, Methodology; Te-I Weng—Supervision, Project administration; Ju-Yu Chen—Writing—Original draft preparation; I-Ting Wang—Resources; Kevin Shu-Leung Lai—Resources; Chia-Mo Lin—Resources; Chu-Yun Teng—Data Curation; Guan-Yuan Chen-Conceptualization, Visualization, Writing—Review & Editing.

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