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An ultra-rapid drug screening method for acetaminophen in blood serum based on probe electrospray ionization-tandem mass spectrometry



Kiyotaka Usui ^{a,*}, Haruka Kobayashi ^a, Yuji Fujita ^b, Eito Kubota ^a,
Tomoki Hanazawa ^c, Tomohiro Yoshizawa ^c, Yoshito Kamijo ^c,
Masato Funayama ^a

^a Division of Forensic Medicine, Tohoku University Graduate School of Medicine, Sendai, 980-8575, Japan

^b Division of Emergency Medicine, Iwate Medical University, Morioka, 020-8505, Japan

^c Emergency Medical Center and Poison Center, Saitama Medical University Hospital, Saitama, 350-0495, Japan

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ABSTRACT

Poisoning incidents caused by drugs, accidental ingestion of poisons, attempted suicide, homicide, and exposure to toxic compounds occur frequently every year across the globe. This raises the need to rapidly identify toxic agents in poisoned patients in a clinical emergency setting. In addition, determining drug/poison concentration is undoubtedly necessary to arrive at a toxicological treatment plan. The purpose of this study was to develop an ultra-rapid drug screening method for the clinical treatment of poisoning. Probe electrospray ionization (PESI), one of the ambient ionization techniques, is able to detect compounds from various biological materials almost directly. We applied the PESI technique to the rapid detection of acetaminophen (APAP). Blood serum samples were diluted 100-fold with 10 mM ammonium formate/ethanol (1:1 v/v) solution including deuterium-labeled internal standards (IS; APAP-d4). Only 10 μ L of the diluted sample was used for measurement. The tandem mass spectrometer (MS/MS) equipped with a PESI was used in selected reaction monitoring mode for the quantitation of APAP; the measurement time was only 18 s. Transitions were set at 152 > 110 for quantitation, 152 > 65 for qualifier, and 156 > 114 for IS (APAP-d4). All measurements were conducted in positive mode. The calibration curve ($1/x^2$) was linear over the range of 1.56–200 μ g/mL ($r^2 = 0.998$), and the limit of detection and quantitation were 0.37 μ g/mL and 1.56 μ g/mL, respectively. The accuracy (bias) and precision (RSD%) of the method were within an acceptable range (–0.15–2.8% and 2.3–6.1%, respectively) and matrix effect at 3 concentrations (95.1–104%) indicated that PESI-MS/MS is only slightly affected by matrices. In real forensic cases, quantitative values of APAP determined by the PESI-MS/MS were almost identical to those determined by the liquid chromatography-MS/MS method. Since PESI-MS/MS is a simple, reliable, and rapid determination method for toxic agents with virtually no need for blood serum pre-treatment, it would be highly suitable for poisoning cases in clinical emergency settings.

* Corresponding author. Division of Forensic Medicine, Tohoku University Graduate School of Medicine, 2-1 Seiryomachi, Aoba-ku, Sendai, 980-8575, Japan. Fax: +81 22 717 8112.

E-mail address: usui@forensic.med.tohoku.ac.jp (K. Usui).

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In the future, a method for simultaneous rapid determination of multiple toxic agents will be developed.

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1. Introduction

Acetaminophen (APAP, popularly known as paracetamol; Fig. 1) is used globally as an analgesic and antipyretic, as it is safe, effective, and results in fewer side effects. Since APAP is mainly used for alleviating fever, shivers, and headache, various over-the-counter drugs (analgesics, multi-ingredient cold medications) contain this drug. Thus, almost everyone has easy access to APAP and hence APAP has been described as one of the drugs most prone to causing an overdose [1,2]. Normally, APAP is mainly excreted in the urine as glucuronide and sulfate conjugate, and partially metabolized to the high-reactive toxic substance, *N*-acetyl-*p*-benzoquinoneimine (NAPQI), by cytochrome p450 enzyme system (mainly CYP2E1) [2,3]. This toxic NAPQI is detoxified by conjugation with glutathione, and then converted to APAP-3-mercapturic acid for urine excretion [2,3]. However, it has been reported that APAP ingestion in large amounts or repeated ingestion exceeding a therapeutic amount, depletes the body's supply of glutathione. In addition, toxic NAPQI causes liver cell necrosis by binding to cellular proteins [2,3]. The reported therapeutic range of serum/plasma APAP concentration is 5–25 µg/mL, 100–150 µg/mL is toxic, and >200 µg/mL is comatose-fatal [4]. Therefore, a method capable of covering these ranges in a single measurement analysis is required. In 1999, a murder case where a victim was repeatedly given an excessive dose of multi-ingredient cold medications (containing APAP) and sake (ethanol) by the perpetrator occurred in Saitama, Japan.

In clinical settings, *N*-acetylcysteine (NAC) is used as an antidote for APAP poisoning. NAC is metabolized to cysteine in the liver and NAPQI is detoxified by this cysteine [2].

It is reported that the risk of liver disorder can be estimated by the Rumack-Matthew nomogram (partially modified by Smilkstein), which is constructed from the blood serum APAP levels and time elapsed after ingestion of APAP [5–8]. Therefore, if blood serum APAP concentration is rapidly and precisely determined, physicians can make an expedited decision if an NAC injection is required.

To quantify APAP in blood serum, liquid chromatography/mass spectrometry (LC/MS) [9], LC-tandem mass spectrometry (LC-MS/MS) [10–12], gas chromatography/MS (GC-MS) [13], and capillary electrophoresis (CE) [14,15] are used to obtain great selectivity, accuracy, and precision. However, most of these techniques are time-consuming and require tedious pre-treatment of blood serum prior to measurements. Therefore, a simple test based on the enzyme-coupled colorimetric method is preferred in a clinical setting [16]. This type of simple test is user-friendly and relatively cost-effective; however, numerous false-positive results have been reported [17–19]. A reliable, automated enzyme immunoassay system has been available, but the time required for the test and the lack of generalization for use in a small medical facility have limited the application of this system [16].

Recently, various ambient MS-based techniques such as DESI (Desorption Electrospray Ionization), paper spray ionization, and swab touch spray have been reported to remove the separation system [20–25]. However, the ease and quickness associated with these techniques have made them suitable for use in point of care testing and critical care. The probe-electrospray ionization (PESI) method developed by Hiraoka is also one of the suitable techniques and can directly analyze liquid samples and solid samples without pre-treatment [26,27].

In the PESI technique, a disposable solid needle is used as a sample probe and an ESI emitter. After the probe needle touches the sample, it moves close to the MS inlet and applies voltage to introduce generated ions. The applications of PESI technique have been reported in various fields such as forensic science, food science, clinical diagnosis, metabolic profiling, and organic synthesis [27–32]. We believe that by using PESI and MS/MS technique in combination, we will be able to develop an ultra-rapid drug determination system for use in a clinical setting.

In this study, we developed a new method by PESI-MS/MS for the ultra-rapid determination of APAP with virtually no pre-treatment.

2. Methods

2.1. Chemicals and reagents

Acetaminophen (purity ≥ 98%) and stable labeled acetaminophen-D₄ solution (100 µg/mL in methanol) were purchased from Sigma–Aldrich (St. Louis, Mo, USA). LC-MS grade methanol, HPLC-grade ethanol, and analytical-grade ammonium formate were purchased from Wako Pure Chemical Industries (Osaka, Japan). A total of 6 lots of frozen, pooled human blood serum were purchased from Cosmo Bio (Tokyo, Japan).

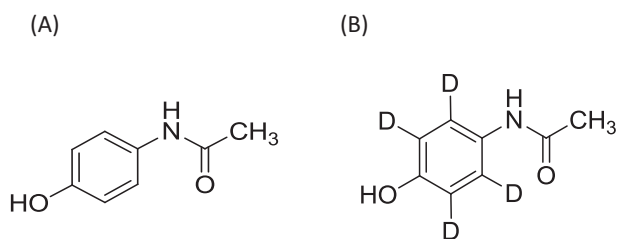


Fig. 1 – Chemical structures of acetaminophen (A) and deuterium-labeled acetaminophen-d₄, the internal standard (B).

2.2. Preparation of standards

Standard stock solution of APAP (10 mg/mL) was prepared in ethanol and stored at -30°C in the dark. The working solutions of APAP were prepared by serial dilution of the stock solution with ethanol. Calibration standards were prepared at concentrations of 1.56, 6.25, 12.5, 25, 50, 100, and 200 $\mu\text{g/mL}$ by spiking blood serum with the appropriate amount of working solutions. The stock solution of acetaminophen- D_4 was diluted with ammonium formate (10 mM):ethanol (1:1 v/v) to a final concentration of 10 ng/mL for use as an internal standard (IS).

2.3. PESI-MS/MS conditions for determination of APAP in blood serum

A Shimadzu 8040 triple quadrupole MS with a PESI ion-source (Shimadzu, Kyoto, Japan) was used for APAP determination (Fig. 2). Blood serum samples were diluted 100-fold with IS solution. A 10- μL sample of diluted blood serum was placed on a dedicated plastic sample plate and set in a PESI ion-source. The probe needle was moved down such that the tip of the needle touched the blood serum sample and then moved up to apply high voltage for ionization. This vertical movement was repeated (3.3 Hz), and the generated ions were introduced into an MS/MS system. The PESI-MS/MS conditions were as follows: probe applied voltage = 1.7 kV, cycle time for probe movement = 150 ms, desolvation line temperature = 250°C , heat block temperature = 50°C and polarity = positive. The probe position (distance from the tip of the needle to the center of the MS inlet) was set at 2 mm for the y-axis and 2.5 mm for the x-axis. For quantitation, the selected reaction monitoring (SRM) transition at m/z 152 > 110 was used for quantitation, and transition at m/z 152 > 65 was used as qualifier ions. The SRM transition at m/z 156 > 114 was used for deuterium-labeled IS. The total analysis time was set to 0.3 min and each point analyzed 5 times.

2.4. LC-MS/MS conditions for detection of APAP in blood serum

Blood serum samples were diluted 1000-fold with IS solution and centrifuged at $18,000\times g$ for 5 min. The supernatant was transferred into a clean vial and 5 μL was injected into an LC-MS/MS system. LC analysis was performed using a Nexera LC system (Shimadzu, Kyoto, Japan). An L-column 2 ODS

column (150 mm \times 1.5 mm i.d.; 5 μm particle size; CERI, Tokyo, Japan), equipped with a guard column (OPTI-GUARD 1 mm C18; Optimize Technologies, Inc., Oregon City, OR, USA), was used for chromatographic separation. The mobile phase consisted of 10 mmol/L ammonium formate (95%) and methanol (5%) (solvent A), and 10 mmol/L ammonium formate (5%) and methanol (95%) (solvent B). The solvent gradient was increased linearly from 0 to 100% solvent B in 15 min and was maintained at this composition for 5 min. Subsequently, the gradient was changed to 0% solvent B and maintained for 10 min to re-equilibrate the column. The flow rate of the mobile phase was set at 0.1 mL/min and the column temperature was maintained at 40°C . MS/MS detection was performed using a QTRAP 5500 system (SCIEX, Framingham, MA, USA). Quantitation was performed in SRM mode. The SRM transition 152 > 110 was used for quantitation, and the transition 152 > 65 was used as qualifier ions. The SRM transition 156 > 114 was used for IS (acetaminophen- D_4). All experiments were conducted in positive ion mode.

2.5. Validation of the method

Seven-point (1.56, 6.25, 12.5, 25, 50, 100, and 200 $\mu\text{g/mL}$) calibration curves were constructed by plotting peak area ratios (APAP/IS) against nominal concentrations of the calibration standards. The curves were fitted using weighted least squares linear regression with a weighting factor of $1/x^2$. Accuracy (bias) and precision of the devised method were determined using quality control samples (QCs) at low (3 $\mu\text{g/mL}$), medium (75 $\mu\text{g/mL}$) and high (150 $\mu\text{g/mL}$) concentrations relative to the calibration range. Each QC sample was analyzed in quintuplicate on 8 consecutive days. Accuracy (bias) was calculated as the percentage deviation from the mean using calculated and nominal concentrations. Intra-day and inter-day precision values were calculated using one-way analysis of variance and expressed as relative standard deviation (RSD%). The limit of detection (LOD) and limit of quantification (LOQ) were determined by the formulae below:

$$\text{LOD} = 3.3 \times \text{SD}/S$$

$$\text{LOQ} = 10 \times \text{SD}/S$$

where SD is the standard deviation of blood serum blank area ($N = 24$) and S is the slope of the calibration curve ($N = 8$). If the calculated LOQ values were lower than the

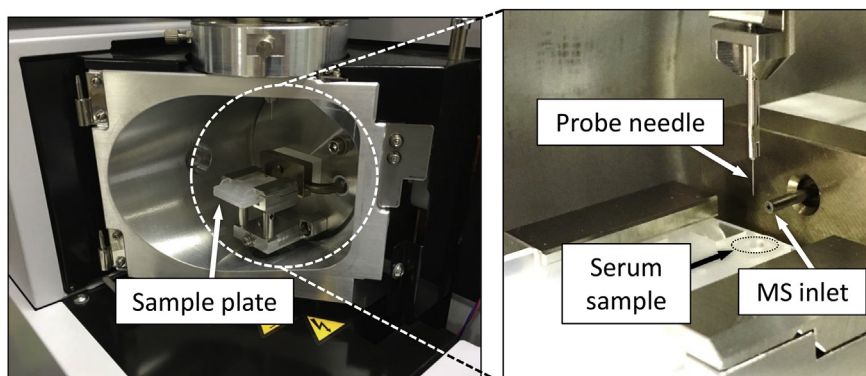


Fig. 2 – Tandem mass spectrometer equipped with a probe electrospray ionization device.

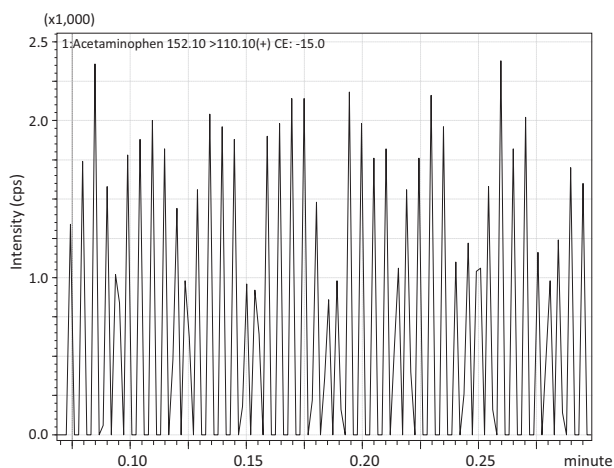


Fig. 3 – Representative SRM current data for acetaminophen (transition 152.1 > 110.1) in blood serum at the limit of quantitation (1.56 µg/mL).

lowest calibrator concentration obtained using the formulae above, the lowest calibrator concentration was defined as the practical LOQ. The matrix effect (ME) was determined using 6 different samples at 3 concentrations: QC low (3 µg/mL), QC medium (75 µg/mL), and QC high (150 µg/mL) and the stability tests were carried out by analyzing QC samples at high and low concentrations. The processed sample stability was determined by analyzing QCs at time intervals of 60 min over 6 h at room temperature. This occurred after 100-fold dilution with ammonium formate (10 mM):ethanol (1:1 v/v). Freeze/thaw stability was determined using 3 cycles of freeze (at –30 °C for 24 h) and thaw (at room temperature). Long-term stability was evaluated by comparing the initial

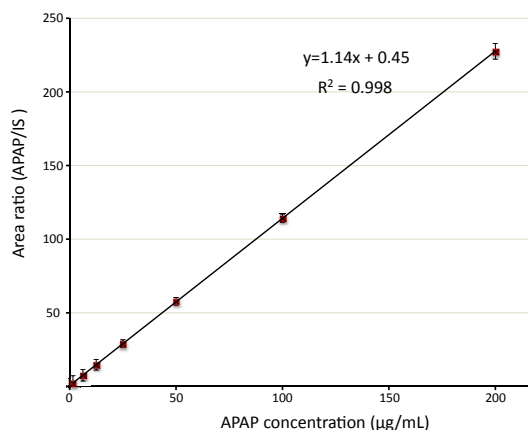


Fig. 4 – Calibration curve for APAP in human blood serum (N = 8).

time sample of QCs to that of the stored sample (at –30 °C for 30 days).

2.6. Application to forensic samples

The devised PESI-MS/MS method was applied to 9 real forensic cases where APAP in blood was already confirmed by LC-MS/MS. We also confirmed that these forensic samples, in addition to containing APAP, had other types of drugs such as psychopharmaceuticals, hypnotics, and cold medicine etc. After APAP concentration was determined, we compared the quantitative values obtained from both PESI-MS/MS and LC-MS/MS.

2.7. Ethical approval

This study was approved by the Ethics Committee of Tohoku University Graduate School of Medicine.

Table 1 – Validation parameters of the method developed.

Calibration range (µg/mL)	1.56–200 (1.56, 6.25, 12.5, 25, 50, 100, and 200)		
r ²	0.998		
LOD (µg/mL)	0.37		
LOQ (µg/mL)	1.56		
QCs (µg/mL)	Precision (RSD%)		Bias (%)
	Intra-day run	Inter-day run	
LOQ (1.56)	3.9	3.6	0.03
Low (3)	3.1	6.1	2.8
Medium (75)	2.3	4.9	2.4
High (150)	2.5	4.2	–0.15
QCs (µg/mL)	Low (3)	Medium (75)	High (150)
Matrix effect % (RSD %)	104 (2.5)	96.7 (2.4)	95.1 (1.8)
	Nominal concentration (µg/mL)	Averaged remaining contents Mean ± SD (%)	
Freeze/thaw stability (3 cycles, –30 °C to RT)	3	99.8 ± 3.1	
	150	98.9 ± 5.0	
Processed sample stability (6.5 h, RT)	3	96.3 ± 8.3	
	150	103 ± 3.9	
Long-term stability (30 days, –30 °C)	3	97.2 ± 6.8	
	150	91.9 ± 3.2	

6 matrices were used.
RT: Room temperature, N = 3.

Table 2 – APAP concentrations determined in forensic cases by using different methods.

APAP conc. ($\mu\text{g/mL}$) by different methods	Case 1	Case 2	Case 3	Case 4	Case 5
PESI-MS/MS	4.80	7.60	13.3	37.8	83.5
LC-MS/MS	4.44	7.20	12.8	38.7	85.2
Other drugs found in blood	Chlorpromazine (20 ng/mL) Levomepromazine (35 ng/mL) Promethazine (44 ng/mL) Amobarbital (10 $\mu\text{g/mL}$) 7-Aminonitrazepam 7-Aminonimetazepam	Etizolam (45 ng/mL) Mirtazapine (37 ng/mL) N-Desalkylflurazepam	Etizolam (54 ng/mL) Levomepromazine (11 $\mu\text{g/mL}$) Promethazine (241 ng/mL) Chlorpheniramine (836 ng/mL) 7-Aminoclonazepam 7-Aminoflunitrazepam	Triazolam (44 ng/mL) Zolpidem (299 ng/mL) Allylisopropylacetylurea	Triazolam (25 ng/mL) Estazolam (590 ng/mL) 7-Aminonitrazepam (470 ng/mL) Aripiprazole (870 ng/mL) Fluvoxamine (2.1 $\mu\text{g/mL}$) Clomipramine (3.4 $\mu\text{g/mL}$) Nortriptyline (6.7 $\mu\text{g/mL}$)
APAP conc. ($\mu\text{g/mL}$) by different methods	Case 6	Case 7	Case 8	Case 9	
PESI-MS/MS	2.28	3.60	4.71	110	
LC-MS/MS	2.29	3.56	4.75	110	
Other drugs found in blood	Triazolam (<5 ng/mL) Brotizolam (<5 ng/mL) Allylisopropylacetylurea Telmisartan Carvedilol	Mirtazapine (3.3 $\mu\text{g/mL}$) Levomepromazine (2.2 $\mu\text{g/mL}$) Flurazepam 1-Ethanolflurazepam 7-Aminoflunitrazepam Lidocain Clomipramine Methylephedrine Dihydrocodeine Chlorpheniramine	Chlorpheniramine Lidocain	Not determined	

3. Results and discussion

3.1. Method validation

Fig. 3 shows the representative total ion current of APAP (1.56 $\mu\text{g/mL}$; the lowest point of calibration curve). Total analysis time was 0.3 min and the method's validation parameters are summarized in Table 1. The linearity of the calibration curve was evaluated 8 times and the curve with a weighting factor of $1/x^2$ showed good linearity ($r^2 = 0.998$) between the concentration range 1.56–200 $\mu\text{g/mL}$ (Fig. 4). The LOD determined using the standard deviation (SD) of blank blood serum ($N = 24$) and the slope of the calibration curve ($\text{LOD} = 3.3 \text{ SD/slope}$), was 0.37 $\mu\text{g/mL}$. The LOQ, the lowest point on the calibration curve, was 1.56 $\mu\text{g/mL}$. The accuracy and precision of LOQ and QCs, low (3 $\mu\text{g/mL}$), middle (75 $\mu\text{g/mL}$) and high (150 $\mu\text{g/mL}$), are also summarized in Table 1 where the accuracies are expressed by the bias (%). The values of bias ranged between -0.15% and 2.8% . The precisions were expressed by the relative standard deviation (RSD %) for intra-day and inter-day run. The RSD% of the intra-day and inter-day run ranged from 2.3% to 3.3%, and 4.2%–6.1%, respectively. The matrix effect corrected by IS (acetaminophen- D_4) gave acceptable values at 3 different concentrations and a total of 6 different matrices was used. The results of each stability test at low and high concentrations are summarized in Table 1. Freeze/thaw stability, processed sample stability, and long-term stability indicated that APAP was stable under the tested conditions.

3.2. Comparison to other methods

Since most patients or victims usually take more than two medicines of differing amounts in clinical or forensic cases, it is important to use a method that is not influenced by other drug and blood serum matrices, to obtain accurate, quantitative values. In this study, although forensic blood serum samples included many different types of drugs, varying amounts of these drugs, and APAP (Table 2), quantitative values of APAP determined by the PESI-MS/MS were almost identical to those determined by the LC-MS/MS method (Table 2). This result indicated that PESI-MS/MS is only slightly affected by other drugs or blood serum matrices.

To date, analytical methods for APAP such as LC-MS/MS and GC-MS have been reported [10–13]. The LOD and LOQ values of these methods were 0.05–0.19 $\mu\text{g/mL}$ and 0.05–1 $\mu\text{g/mL}$, respectively. The LOD and LOQ values of PESI-MS/MS ($\text{LOD} = 0.37 \mu\text{g/mL}$; $\text{LOQ} = 1.56 \mu\text{g/mL}$) were slightly higher than those of other methods because blood serum was diluted 100-fold in the PESI-MS/MS method. The reported therapeutic range of serum/plasma APAP concentration is 5–25 $\mu\text{g/mL}$, 100–150 $\mu\text{g/mL}$ is toxic, and $>200 \mu\text{g/mL}$ is comatose-fatal [4]. Since our devised method covered these ranges, we believe it is applicable for practical use.

Infusion system is another technique that shortens analysis time. Since the blood serum is directly and continuously introduced into the ion-source in the infusion system, the mass spectrometer is contaminated by blood serum and requires frequent system cleaning to ensure sensitivity is

maintained. Furthermore, users must carefully clean the syringe and PEEK (polyether ether ketone) tubes to avoid blood contamination between measurements. The process of direct injection is therefore tedious and time-consuming. On the other hand, in the PESI system, direct introduction of blood serum into the ion-source is not performed. As the tip of the probe needle touches the sample (only a few pL sample is captured) and only ionized compounds are introduced into the ion-source, the possibility of contamination is minimized. In addition, probe needles and sample plates are disposable, minimizing the risk of contamination.

PESI-MS/MS is suggested to be suitable in clinical settings, as inexperienced operating personnel conducting a toxicological analysis can acquire reliable and quantitative values easily and rapidly. In addition, as a disposable sample holder and probe needle are used, there is virtually no risk of contamination. Through the use of the PESI-MS/MS technique, we can conveniently determine the effectiveness of medical treatment by ultra-rapid measurements of drug/poison concentration in blood serum. Ultimately, PESI-MS/MS could enable the simultaneous, rapid determination of these compounds in blood serum.

PESI-MS/MS, however, has some disadvantages. Since these ambient-MS techniques do not have chromatographic separation systems, the compounds which have the same precursor ions such as isobars/isomers result in mixed product ion spectra. Another issue is the cost associated with introducing the tandem mass spectrometer. The large space required to house the spectrometer and its peripheral equipment (e.g., nitrogen generator and air compressor or gas cylinders) is yet another issue in a clinical setting. Therefore, the development of a miniature mass spectrometer and reduction in the cost of introduction would be required for the practical use of PESI-MS/MS in a clinical setting.

Conflicts of interest statement

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