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# Direct quantification of creatinine in human urine by using isotope dilution extractive electrospray ionization tandem mass spectrometry

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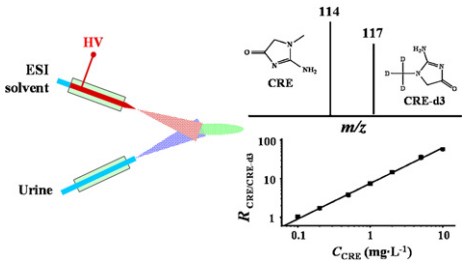
## HIGHLIGHTS

- High throughput analysis of urinary creatinine is achieved by using ID-EESI–MS/MS.
- Urine sample is directly analyzed and no sample pre-treatment is required.
- Accurate quantification is accomplished with isotope dilution technique.

## GRAPHICAL ABSTRACT

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## ABSTRACT

Urinary creatinine (CRE) is an important biomarker of renal function. Fast and accurate quantification of CRE in human urine is required by clinical research. By using isotope dilution extractive electrospray ionization tandem mass spectrometry (EESI–MS/MS) a high throughput method for direct and accurate quantification of urinary CRE was developed in this study. Under optimized conditions, the method detection limit was lower than 50 μg L$^{-1}$. Over the concentration range investigated (0.05–10 mg L$^{-1}$), the calibration curve was obtained with satisfactory linearity ($R^2$ = 0.9861), and the relative standard deviation (RSD) values for CRE and isotope-labeled CRE (CRE-d3) were 7.1–11.8% ($n$ = 6) and 4.1–11.3% ($n$ = 6), respectively. The isotope dilution EESI–MS/MS method was validated by analyzing six human urine samples, and the results were comparable with the conventional spectrophotometric method (based on the Jaffe reaction). Recoveries for individual urine samples were 85–111% and less than 0.3 min was taken for each measurement, indicating that the present isotope dilution EESI–MS/MS method is a promising strategy for the fast and accurate quantification of urinary CRE in clinical laboratories.



## 1. Introduction

Acute renal failure (ARF) can result in a mortality rate of 50% and the relevant costs are about 8 billion dollars per year [1–3]; however, the early diagnosis of ARF can greatly reduce the mortality rate and the associated costs. Creatinine (2-amino-1,5-dihydro-1-methyl-4H-imidazol-4-one, CRE) is generally monitored as a biomarker of renal function in clinical diagnosis and also suggested to be the clinical marker for the early detection of ARF. As the end metabolite derived from the non-enzymatic conversion of creatine and phosphocreatine, CRE is removed from the blood by the kidneys and excreted into the urine at a fairly constant rate (25 mg per kg of body weight per day).

Due to the importance of CRE in clinic research, a number of analytical methods have been developed for urinary CRE

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detection, including: spectrophotometric method based on the Jaffe reaction [4], enzymatic method [5], capillary zone electrophoresis [6], surface-enhanced Raman spectroscopy (SERS) [1], high performance liquid chromatography (HPLC) [7,8], high performance thin-layer chromatography (HPTLC) [9], liquid chromatography tandem mass spectrometry (LC–MS/MS) [10,11] and gas chromatography mass spectrometry (GC–MS) [12,13]. Among these methods, the spectrophotometric method (based on the Jaffe reaction) is the oldest and most widely used method whereas this method is adversely affected by endogenous interferences [14,15]. With the increasing application of LC–MS/MS in clinical laboratories, LC–MS/MS has been recommended as a reference technology of choice for monitoring urinary CRE due to its advantages in sensitivity and specificity [11,16].

Recently, the efficiency of MS-based methods has been dramatically improved by using ambient ionization techniques [17], such as desorption electrospray ionization (DESI) [18], direct analysis in real time (DART) [19], desorption atmospheric pressure chemical ionization (DAPCI) [20], dielectric barrier discharge ionization (DBDI) [21], low-temperature plasma probe (LTP) [22], atmospheric solids analysis probe (ASAP) [23], laser ablation with electrospray ionization (LAESI) [24] and extractive electrospray ionization (EESI) [25]. Among all these ambient ionization techniques, EESI is characterized for rapid analysis of complex liquid samples [26]. In an EESI source, two independent sprayers are used to produce charged microdroplets of the primary ESI solvent by electrospray and neutral microdroplets of the sample solution by pneumatic nebulization; with the collision between the charged and neutral microdroplets, target compounds in the sample solution are extracted into the charged droplets and ionized subsequently [27,28]. Due to the extractive ionization process in EESI, ion suppression effects can be reduced even when complex liquid matrices are directly injected (*e.g.*, urine, beer and edible oil); this advantage of EESI enables the direct and fast analysis of complex liquids without pre-treatment and/or chromatographic separation (*e.g.*, LC and GC) [25,29,30].

Isotope dilution is a widely used strategy coupled to MS-based methods, *e.g.*, LC–MS [31], GC–MS [32], in which isotope labeled standards are added into the sample prior to sample pre-treatment and/or instrumental analysis. By using the isotope dilution method, loss, contamination, errors, *etc.*, occurred during sample pre-treatment and/or instrumental analysis can be tracked and corrected regarding that the physicochemical properties of the native compounds are quite similar to their labeled counterpart. Thus, quantification accuracy and precision can be improved.

In this study, an isotope dilution EESI–MS/MS method for fast and accurate quantification of CRE in human urine was developed by using a homemade EESI source coupled to an ion trap mass spectrometer. EESI and MS/MS analysis conditions were systematically studied for qualitative and quantitative detection of urinary CRE; the developed isotope dilution EESI–MS/MS method was validated by the conventional spectrophotometric method (based on the Jaffe reaction). The results obtained in the present work indicate that the proposed isotope dilution EESI–MS/MS method is a promising strategy for high throughput and accurate analysis of CRE in human urine.

## 2. Materials and methods

### 2.1. Chemicals and materials

CRE was purchased from Sigma–Aldrich (St. Louis, MO, USA) and CRE-d3 (methyl-d3) was obtained from Toronto Research Chemicals Inc. (Toronto, Canada). Methanol (MeOH) (HPLC grade) was provided by Burdick & Jackson (Muskegon, MI, USA). Ethanol (EtOH), 1-propanol (PrOH), 1-butanol (BuOH), 1-pentanol (PeOH) and 1-hexanol (HeOH) (GC standards) were bought from Tianjin Guangfu Fine Chemical Research Institute (Tianjin, China). Acetic acid (HAc) was supplied by Guangzhou Xilong Chemical Co. Ltd. (Guangzhou, China). Ultrapure water (resistivity 18.2 MΩ cm) was supplied by a Barnstead Nanopure ultrapure water purification system (Thermo Scientific, USA). All chemicals were directly used without further treatment.

### 2.2. Preparation of standard solutions and spiked urine samples

Stock solutions of CRE (100 mg $L^{-1}$) and CRE-d3 (100 mg $L^{-1}$) were prepared by dissolving 10.0 mg of CRE or CRE-d3 in 100 mL of ultrapure water and stored in 20-mL brown vials in the dark at 4 °C. A series of CRE aqueous standard solutions (0–10 mg $L^{-1}$) were prepared by serially diluting the CRE stock solution with ultrapure water; the CRE-d3 concentration in each standard solution was 100 μg $L^{-1}$.

For the spiked urine samples, the raw urine sample was firstly diluted by 1:500 with ultrapure water while the dilution factor was determined in the pre-experiments. Then, five aliquots of the urine sample were spiked with CRE and CRE-d3. The spike CRE concentrations in individual spiked urine samples were 0.5, 1, 1.5, 2 and 4 mg $L^{-1}$, respectively, and the spike CRE-d3 concentration was always 1 mg $L^{-1}$. More details were supplied in the Electronic Supplementary Information (Table S1).

It is noteworthy the CRE content in the spiked urine is 1.5–3 times the CRE content in the unspiked urine according to the standard addition method. The standard addition method was adopted for the quantification of urinary CRE instead of the external calibration method in this study, because the standard addition method can solve the problem of matrix effects caused by the direct analysis of urine samples.

The real raw urine samples were donated by seven healthy male and female volunteers (24–26 years old). A portion of each raw urine sample was collected in a 20-mL brown vial and stored in the dark at −20 °C before use.

Both standard solutions and spiked urine samples were freshly prepared each experimental day.

### 2.3. EESI–MS/MS analysis

A homemade EESI source (Fig. S1) was set up as described previously [33]. In brief, the distance $a$ and angle $\alpha$ between the end-tips of two sprayers was about 1 mm and 60°, respectively. The distance $b$ and angle $\beta$ between the end-tip of the ESI solvent/sample sprayer and the MS inlet was about 10 mm and 150°, respectively.

The homemade EESI source was coupled to a commercial LTQ-XL mass spectrometer (Finnigan, San Jose, USA). The mass spectrometer worked in the positive ion detection mode. The ESI voltage was set at +3.5 kV. The temperature of the ion-transport capillary was 150 °C. The injection rates of the primary ESI solvent and sample solution were 3 μL $min^{-1}$ and 5 μL $min^{-1}$, respectively. For nebulizing ESI solvent and sample solution, high purity nitrogen gas (purity ≥ 99.999%) was supplied from a gas cylinder and the pressure was set at 0.8 MPa.

Regarding MS/MS analysis, collision induced dissociation (CID) experiments were carried out. The protonated molecular ion $[M+H]^+$ of CRE ($m/z$ 114) or CRE-d3 ($m/z$ 117) was selected as the precursor ion. Helium gas was used as the collision gas and the pressure in the collision cell ranged from 0.84 to 0.98 × $10^{-5}$ Torr. The isolation width and activation time were set at 2.0 Da and 30 ms, respectively. The MS/MS spectra were recorded over the $m/z$ range of 15–200. The automatic gain control (AGC) was enabled to regulate the number of ions injected in the cell; meanwhile the maximum ion injection time (the maximum time that ions are

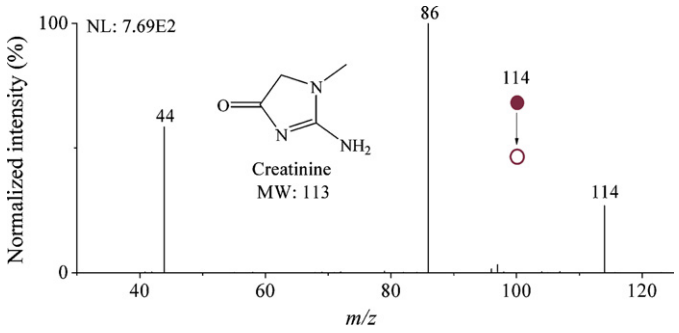


**Fig. 1.** EESI–MS/MS spectrum of CRE protonated molecular ion at $m/z$ 114 obtained by analyzing a CRE standard solution (100 μg $L^{-1}$) when NCE = 25% at AQ = 0.30.

allowed to accumulate in the mass analyzer when AGC is on) was set at 200 ms.

Before each experiment, 100 μg $L^{-1}$ of CRE standard solution was analyzed by EESI–MS/MS to ascertain that the homemade EESI source works properly. For each sample, at least six independent measurements were carried out.

### 2.4. Spectrophotometric analysis

For validating the developed isotope dilution EESI–MS/MS method, the urine samples were also analyzed by the conventional spectrophotometric method based on the Jaffe reaction according to the WS/T 97-1996 method.

## 3. Results and discussion

### 3.1. Optimization of MS/MS analysis conditions

MS/MS analysis was performed for qualitative and quantitative detection of CRE. MS/MS analysis is required when the EESI source is used. This is because urine samples have been directly introduced into the source without pre-treatment and chromatographic separation, and matrix compounds can result in false positive peaks in MS spectra. However, these false positive results can be excluded by MS/MS analysis.

MS/MS analysis was achieved *via* CID experiments. Two operating parameters in CID, *i.e.*, activation Q (AQ) and normalized collision energy (NCE) were systematically optimized by analyzing a CRE aqueous solution (100 μg $L^{-1}$). AQ is a factor correlated to the trapping and fragmentation effectiveness [34,35]; NCE scales the amplitude of the voltage applied to the ions, and this voltage can change the translational kinetic energy of the ions in the ion trap [36].

As shown in Figs. S2 and S3, when AQ was set at 0.10 and 0.20, protonated creatinine $[M+H]^+$ ($m/z$ 114) was ineffectually fragmented at NCE 0–100%. As AQ was further increased to the values of 0.25 and 0.30, two fragment ions were observed at $m/z$ 44 and $m/z$ 86 at NCE ≥20% (Fig. 1), which were assigned as $[CH_3NCH_3]^+$ [13] and $[M+H-CO]^+$, respectively. When AQ ranged from 0.35 to 0.60, CRE was still fragmented at NCE ≥ 20%; however, only the peak at $m/z$ 86 remained in the MS/MS spectra and the fragment ion at $m/z$ 44 could not be observed (Fig. S2). This is because the stable ion at the lowest $m/z$ in the ion trap during activation is limited to the AQ, and the lowest $m/z$ can be approximated by the following expression:

$$\text{Lowest } m/z = \frac{[\text{Parent Mass}] \times [\text{AQ}]}{0.908} \quad (1)$$

Thus, according to Eq. (1) shown above, the ion at $m/z$ 44 was not stable in the ion trap at AQ 0.35–0.60. Similarly, at AQ ≥ 0.65, the fragment ion at $m/z$ 86 could not be trapped in the cell neither (Fig. S2). As a result, the value of AQ is not only related to the fragmentation effectiveness of the parent ion but also the fragment ions that can be obtained.

Moreover, when AQ was raised from 0.25 (instrument default value) to 0.35, the signal intensity at $m/z$ 86 was increased by a factor of 6 when NCE was set at 25% at individual AQ values (Fig. S3). This result indicated that the parent ion ($m/z$ 114) was more effectively fragmented when AQ was set at the values rather than the instrument default value. Therefore, the optimization of AQ is as necessary as NCE, although in most previous studies only NCE has been systematically optimized.

Thus, in MS/MS analysis CRE can be effectively fragmented when NCE ≥ 20% at individual AQ values in the range of 0.25–0.70. The fragment ions at $m/z$ 44 and/or $m/z$ 86 can be obtained only at AQ ranging from 0.25 to 0.60. In addition, because the intensity of the fragment ion at $m/z$ 86 was higher than $m/z$ 44 (Fig. 1), the ion at $m/z$ 86 was selected for the optimization of EESI conditions and quantification of urinary CRE in the following experiments. The optimum AQ and NCE for detecting the signal at $m/z$ 86 was 0.35 and 25% (Fig. S3), respectively. The EESI–MS/MS spectrum obtained under optimized CID conditions was presented in Fig. S4.

### 3.2. Optimization of EESI conditions

The extraction and ionization processes were optimized by adjusting the primary ESI solvent composition, ESI voltage, ESI solvent injection rate, sample solution injection rate, ion-transport capillary temperature, ESI voltage and sheath gas pressure.

For optimizing the primary ESI solvent composition, seven solvents were tested, including water, MeOH, EtOH, PrOH, BuOH, PeOH and HeOH. These solvents are of different polarities, and thus will perform differently in the extraction and ionization processes. The diluted urine sample (1:10) was used as the sample solution, and thus ionization suppression from matrix compounds (*e.g.*, concentrations of sodium chloride, urea and hippurate in urine are 8.0, 13.4 and 1.25 g $L^{-1}$, respectively [37]) can be taken into consideration.

The highest signal intensity was achieved when MeOH was used as the primary ESI solvent (Fig. S5). Different EESI–MS mass spectra (over the $m/z$ range of 15–200) of the urine sample were obtained when water, MeOH, EtOH, PrOH, BuOH, PeOH or HeOH was used as the primary ESI solvent (Fig. S6). The differences in the MS spectra implied the different extraction abilities of different primary ESI solvents. However, these differences were not further discussed here, since the present study has been focused on the urinary CRE detection.

Additionally, to further improve the ionization efficiency, HAc was added into the primary ESI solvent of MeOH as the supporting electrolyte. However, no significant increase in signal intensity was observed when 0.1% (V/V) HAc was added; the signal intensity was decreased by a factor of 2 when the HAc content was continually increased to 1% and 3% (V/V) (Fig. S7). Consequently, the optimum primary ESI solvent composition was finally determined as MeOH without the addition of HAc.

Furthermore, other EESI conditions, *i.e.*, the ESI solvent injection rate, ESI voltage, ion-transport capillary temperature and sheath gas pressure were optimized as 1 μL $min^{-1}$, +4 kV, 400 °C and 1.0 MPa, respectively (Figs. S8–S11). As for the sample injection rate, although higher signal intensity was obtained at higher injection rate, the rate was finally set at 5 μL $min^{-1}$ (Fig. S12), to minimize the contamination of the mass spectrometer.

### 3.3. Quantification of urinary CRE

As for CRE quantification, CRE-d3 was used as the isotopic internal standard to correct for variations in CRE concentration caused by the instabilities of the homemade EESI source, MS instrument

**Table 1**
Standard addition curve, $R^2$, $RSD_{CRE}$ and $RSD_{CRE\text{-}d3}$ for six urine samples.

| No.[a] | Standard addition curve | $R^2$ | $RSD_{CRE}$ (%) ($n$ = 6)[b] | $RSD_{CRE\text{-}d3}$ (%) ($n$ = 6)[c] |
|---|---|---|---|---|
| V-1 | $Y = 1.280X + 1.04$ | 0.9888 | 4.3–9.3 | 2.9–7.5 |
| V-2 | $Y = 1.148X + 1.13$ | 0.9821 | 4.0–10.6 | 5.9–11.6 |
| V-3 | $Y = 1.352X + 1.64$ | 0.9951 | 4.2–7.8 | 2.4–9.3 |
| V-4 | $Y = 1.049X + 1.96$ | 0.9772 | 4.3–8.9 | 2.5–8.8 |
| V-5 | $Y = 1.485X + 1.59$ | 0.9868 | 5.2–14.3 | 6.8–7.8 |
| V-6 | $Y = 1.388X + 1.11$ | 0.9748 | 4.3–8.2 | 2.7–14.6 |

[a] The capital letter "V" stands for the word of "volunteer", and V-1–V-3 are female volunteers while V-4–V-6 are male volunteers.
[b] $RSD_{CRE}$ is the RSD for CRE.
[c] $RSD_{CRE\text{-}d3}$ is the RSD for CRE-d3.

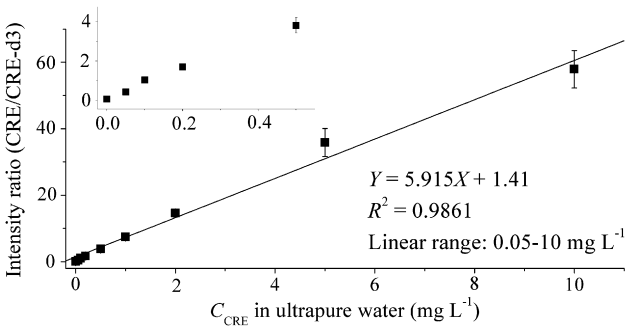


**Fig. 2.** Dependence of CRE signal intensity on CRE concentration in ultrapure water.

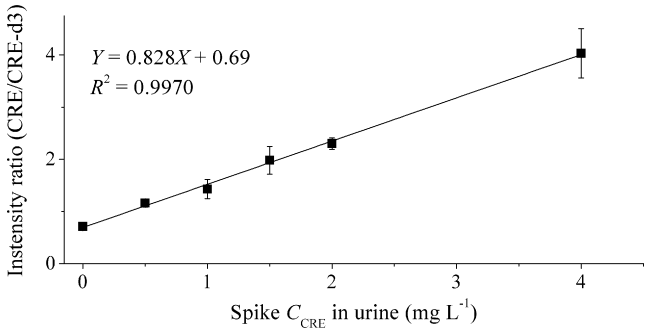


**Fig. 3.** Quantification of CRE concentration ($C_{CRE}$) in human urine by using the standard addition method.

and long-term operation. CRE-d3 was quantified by monitoring its fragment ion $[M+H-CO]^+$ ($m/z$ 89) obtained by EESI–MS/MS analysis (Fig. S13).

For CRE aqueous standard solutions (0–10 mg L$^{-1}$), the intensity ratio of CRE and CRE-d3 was plotted as a function of CRE concentration in the standard solutions (Fig. 2) while the variation of intensity ratio with CRE concentration in the low-concentration range (0–0.5 mg L$^{-1}$) were presented in the inset of Fig. 2. When the limit of detection (LOD) is defined as the CRE concentration giving the signal intensity three times the blank sample, the LOD for aqueous CRE was lower than 50 μg L$^{-1}$ (inset in Fig. 2). Within the concentration range investigated (0.05–10 mg L$^{-1}$), the calibration curve was obtained with satisfactory linearity ($R^2$ = 0.9861). The values of the relative standard deviation (RSD) for CRE were 7.1–11.8% ($n$ = 6) and for CRE-d3 were 4.1–11.3% ($n$ = 6).

For urinary CRE quantification, the spiked urine samples were analyzed together with the unspiked one. It should be noted that the raw urine samples were 1:500 diluted before being spiked, for the concentration of CRE in the raw urine is approximately 1.3–1.5 g L$^{-1}$ [13,37] and greatly exceeds the linear range obtained (Fig. 2).

The intensity ratio of CRE and CRE-d3 was linearly related to the spike CRE concentration in the urine samples ($R^2$ = 0.9970); the values of RSD for urinary CRE and CRE-d3 were 4.9–13.4% ($n$ = 6) and 4.3–6.3% ($n$ = 6), respectively. According to the standard addition curve, the urinary CRE concentration was determined as 417 mg L$^{-1}$ (Fig. 3). On the other hand, the same urine sample was also analyzed by the spectrophotometric method and the CRE concentration was measured as 367 mg L$^{-1}$.

The recovery ($R$) of CRE was calculated based on the equation shown below:

$$R(\%) = \left[\frac{C_{CRE}(\text{EESI–MS/MS})}{C_{CRE}(\text{Spectrophotometry})}\right] \times 100 \quad (2)$$

where $R$ is the recovery of urinary CRE, $C_{CRE}$ (EESI–MS/MS) is the CRE concentration detected by the isotope dilution EESI–MS/MS method and $C_{CRE}$ (Spectrophotometry) is the CRE concentration measured by the spectrophotometric method. According to Eq. (2), the $R$ was determined as 114%.

Thus, the above results indicated that the direct and accurate measurement of urinary CRE without pre-treatment and chromatographic separation was successfully achieved with satisfactory accuracy and precision by using isotope dilution EESI–MS/MS.

### 3.4. Validation of the isotope dilution EESI–MS/MS method

The developed isotope dilution EESI–MS/MS method was further validated by analyzing the real urine samples donated by six healthy volunteers.

The spiked and unspiked urine samples were analyzed with isotope dilution EESI–MS/MS, and the standard addition curves were obtained for individual urine samples with reasonable linearity and RSD (Table 1). $R^2$ was in the range of 0.9748–0.9951, and the RSD values were 4.0–14.3% ($n$ = 6) for CRE and 2.4–14.6% ($n$ = 6) for CRE-d3. Moreover, the CRE levels in individual urine samples were determined by the standard addition curves (the isotope dilution EESI–MS/MS method) (Fig. S14) and the external standard curve (the spectrophotometric method), respectively (Table 2). Satisfactory recoveries were obtained for individual urine samples, which were in the range of 85–111% except for the sample from volunteer 4 (149%). Less than 0.3 min was taken for each measurement of urinary CRE and six independent measurements of one sample were accomplished within 5.0 min.

**Table 2**
Quantification of CRE in six real human urine samples by the isotope dilution EESI–MS/MS and spectrophotometric methods, respectively.

| No. | $C_{CRE}$ (mg L$^{-1}$)[a] | $C_{CRE}$ (mg L$^{-1}$)[b] | $R$ (%) |
|---|---|---|---|
| V-1 | 406 | 473 | 86 |
| V-2 | 492 | 442 | 111 |
| V-3 | 607 | 559 | 109 |
| V-4 | 934 | 626 | 149 |
| V-5 | 535 | 630 | 85 |
| V-6 | 1599 | 1733 | 92 |

[a] Data were obtained by the isotope dilution EESI–MS/MS method.
[b] Data were obtained by the spectrophotometric method.

With these acceptable quantification results, the procedures of sample pre-treatment and chromatographic separation (*e.g.*, LC) have been obviated in the present analytical strategy, and the analytical time (<0.3 min) is either comparable to or shorter than those by using the LC–MS methods (*e.g.*, 0.59 min [11] and 12.4 min [10]). Although the values of the intra-day coefficient of variation (CV) of the standard solutions in this work (7.1–11.8%) are higher compared with the results from the LC–MS methods (*e.g.*, 1.5–8.5% [10], <8.0% [11]), the accuracy and reproducibility of the proposed method can be further improved with the improvement on the fabrication of the present homemade EESI source.

## 4. Conclusions

In this study, by using isotope dilution EESI–MS/MS, a direct and accurate quantitative method for detecting CRE in human urine without pre-treatment and chromatographic separation was successfully developed and fully validated. This is the first time to apply EESI–MS/MS together with the isotope dilution technique for the accurate quantification of the target compound in a complex biological liquid matrix. With the application of the method developed, fast quantitative detection of urinary CRE was satisfactorily demonstrated (<0.3 min for each measurement). The results obtained in our study suggest that isotope dilution EESI–MS/MS is a promising strategy for the high throughput and accurate quantification of urinary CRE without pre-treatment and chromatographic separation. Moreover, it can be speculated that the accuracy and reproducibility of the proposed method can be further improved with the improvement on the fabrication of the homemade EESI source. Besides, the on-site and real-time detection of urinary CRE can also be realized provided the EESI source is combined to a miniature ion trap mass spectrometer, and this will essentially facilitate the point-of-care testing for kidney diseases such as ARF.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aca.2012.08.040.

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