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Technical Note

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Direct Determination of Urinary Creatinine by Reactive-Thermal Desorption-Extractive Electrospray-Ion Mobility-Tandem Mass Spectrometry.

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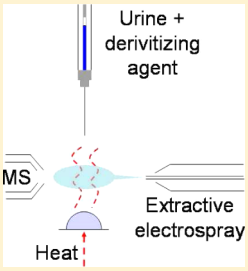
ABSTRACT: A direct, ambient ionization method has been developed for the determination of creatinine in urine that combines derivatization and thermal desorption with extractive electrospray ionization and ion mobility-mass spectrometry. The volatility of creatinine was enhanced by a rapid on-probe aqueous acylation reaction, using a custom-made thermal desorption probe, allowing thermal desorption and ionization of the monoacylated derivative. The monoacyl creatinine $[M + H]^+$ ion (m/z 156) was subjected to mass-to-charge selection and collision induced dissociation to remove the acyl group, generating the protonated creatinine $[M + H]^+$ product ion at m/z 114 before an ion mobility separation was applied to reduce chemical noise. Stable isotope dilution using creatinine-d_3 as internal standard was used for quantitative measurements. The direct on-probe derivatization allows high sample throughput with a typical cycle time of 1 min per sample. The method shows good linearity (R^2 = 0.986) and repeatability (%RSD 8−10%) in the range of 0.25−2.0 mg/mL. The creatinine concentrations in diluted urine samples from a healthy individual were determined to contain a mean concentration of 1.44 mg/mL creatinine with a precision (%RSD) of 9.9%. The reactive ambient ionization approach demonstrated here has potential for the determination of involatile analytes in urine and other biofluids.



Thermal desorption is a commonly used method for the generation of gaseous phase analytes from less volatile matrix components. The process of directly heating a sample to liberate molecules from the liquid phase has been widely applied to the analysis of volatile organic compounds (VOCs) by headspace gas chromatography (GC).[1] Other applications of thermal desorption include headspace analysis by mass spectrometry (MS)[2] and narcotic/explosive detection by ion mobility (IM) spectrometry.[3] The application of thermal desorption is limited by the volatility of the target analyte, which can be altered by specific derivatization reactions.[4−6]

The substitution reactions of alkylation, acylation, and silylation[5] can significantly reduce the temperatures required for thermal desorption of a molecule. However, these reactions are generally carried out in a nonaqueous environment. The process of removing the water from a sample prior to derivatization enables a wide range of derivatization reactions to be carried out but significantly increases the sample preparation time required for each analysis. The use of aqueous based derivatization reactions overcomes the difficulties associated with aqueous samples, allowing direct derivatization.[6] The use of aqueous derivatization reactions for the detection of compounds in urine has been an area of interest, particularly in the analysis of drugs of abuse,[7,8] but the technique is still in its infancy for biomedical prognostic/diagnostic applications.[9]

The direct analysis of urine by electrospray ionization mass spectrometry is problematic, because of ion suppression effects arising from the high salt content of urine.[10] Prior separation by high performance liquid chromatography (HPLC) or GC, hyphenated with MS, is therefore usually used for the detection of excreted metabolites relating to disease states such as lung disorder, diabetes, and cancer.[11−13] It is common practice in the determination of urinary metabolites to normalize the target molecule concentration to the excretion of creatinine.[14] The normalization process allows the absolute amount of an excreted metabolite to be expressed in relation to the creatinine level (i.e., μg/g creatinine) which can then be used as a comparison against a healthy population. The concentration of creatinine in both urine and blood serum is dependent on several variables, including the glomerular filtration rate, which is itself dependent on age, gender, and body size.[15,16] The measurement of the creatinine clearance rate (CrCl), the amount of creatinine passed in 24 h, determines the glomerular filtration rate (GFR) which also provides information on kidney function.

A variety of methods are available for the determination of creatinine (**I**, below) in urine, the most widely used being the Jaffe reaction.[17]

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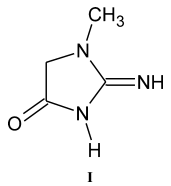

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This colorimetric based approach places serum or urine in an alkaline environment to facilitate the reaction between creatinine and picric acid to form red/orange crystals. The poor specificity of the Jaffe reaction limits its use, particularly in serum, and has therefore been modified numerous times to increase the specificity of the method.[18] Other methods include a two-step enzymatic digestion of creatinine,[19] which has higher selectivity compared to the Jaffe reaction but is limited by a complex multiple step sample preparation procedure and involves ion exchange chromatography.[20] Creatinine determination in complex biofluids has been previously performed by GC/MS with isotope dilution, using a derivatization step to increase analyte volatility.[21] The direct determination of urinary creatinine levels by extractive electrospray (EESI)-tandem mass spectrometry has also been demonstrated,[22] showing that ambient ionization can be used to measure urinary creatinine with high sensitivity. However, this approach does not separate the target analyte from the urine matrix prior to ionization and may be susceptible to interference and ion suppression effects. Thermal desorption of a volatile target molecule allows ionization by EESI[23] while discriminating against the urinary salts and other underivatized metabolites. Here, the combination of a rapid aqueous phase derivatization to promote desorption of an involatile analyte directly from the urine matrix, combined with EESI and ion mobility mass spectrometry (IM-MS) of the volatile derivative, is demonstrated for the determination of creatinine. This approach has wider application as an ambient ionization method for the rapid, direct quantitation of involatile urinary analytes unaffected by ion suppression from salts and other components.

EXPERIMENTAL SECTION

Chemicals. Analytical grade water and methanol were purchased from Fisher Scientific (Loughborough, UK). HPLC grade formic acid, acetic anhydride (99%), and creatinine (98%) were purchased from Sigma Aldrich (Gillingham, UK). Creatinine-d_3 was purchased from Toronto Research Chemicals Inc. (Ontario, Canada). All chemicals were used without further purification.

AP/TD Probe. A schematic diagram of the ion source region of a Waters Synapt HDMS spectrometer (Waters, Manchester, UK), modified in-house for direct on-probe reactive derivatization (R-TD) and extractive electrospray is shown in Figure 1.[24] The TD probe was constructed by placing a cylindrical heater cartridge (2.5 mm o.d. × 50 mm; RS Components Ltd., Northants, UK) into a stainless steel tube (12 i.d. × 300 mm) with the heater positioned inside an aluminum adaptor at the probe tip. Samples and the derivatization reagent were injected into a brass sample holder (2.5 mm o.d. × 10 mm) located within the aluminum adaptor, with the base of the sample holder in direct contact with the heater cartridge. The sample holder was inserted through a hole in the side of a stainless steel tube (12 mm o.d. × 50 mm) located between the ESI probe and the mass spectrometer inlet cone that spatially confines the thermally desorbed analytes for ionization by an in-line electrospray plume. The cartridge temperature was regulated

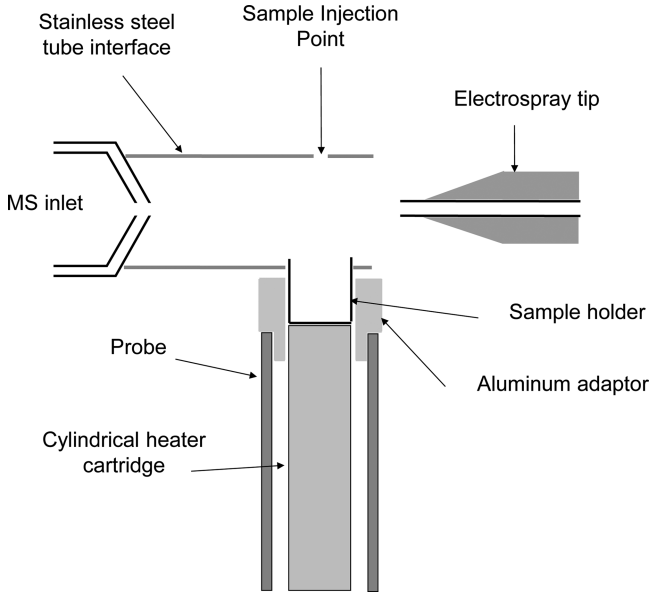

Figure 1. Schematic diagram of the R-TD-EESI-MS ion source region (not to scale).

using a temperature controller (Tempatron, Reading UK) to set the sample holder temperature to 100 °C.

Sample Preparation. Creatinine standards were prepared in water at concentrations in the range of 2.5−20 μg/mL (60 μL). Urine samples were diluted with water (1:100), and deuterated creatinine-d_3 was added at a concentration of 5 μg/mL (40 μL). In preliminary creatinine acylation experiments, urine was mixed with acetic anhydride (100 μL) at a 1:1 ratio to initiate the acylation reaction. The samples were continually agitated, and aliquots (10 μL) of the reaction mixture were removed, diluted in 50:50 methanol/water (1:10) with 0.1% formic acid, and directly infused into the ESI source of the mass spectrometer. On-probe derivatization was carried out by the direct injection of a mixture of diluted urine (10 μL of a 1:100 dilution in water), containing the creatinine-d_3 internal standard, and acetic anhydride (10 μL) into the sample holder of the TD probe, which was preheated to 100 °C.

EESI-IM-MS/MS Conditions. The EESI solvent consisted of 50:50 MeOH/H_2O (v/v) with 0.1% formic acid (v/v), which was infused from a syringe pump at a flow rate of 10 μL/min. The mass spectrometer (Synapt HDMS, Waters, Manchester, UK) was operated in sensitivity (V) mode using positive ionization with a capillary voltage of 2.8 kV and a cone voltage of 15 V; a mass range of m/z 50−500 was monitored. The source temperature was set to 120 °C, with a nebulizer gas flow (N_2) of 60 L/h. The quadrupole was set to transmit the monoacylated creatinine ($[M + H]^+ = m/z$ 156) and creatinine-d_3 ($[M + H]^+ = m/z$ 159) ions. The trap region of the Waters Tri-Wave cell was set to 13 V for collision induced dissociation of the mass-selected precursor ions using N_2 as the collision gas. The traveling wave ion mobility (TWIM) cell wave height was 6 V; the wave velocity was 300 m/s, and the N_2 drift gas was set to 30 mL/min. The TOF pusher pulse interval was 45 μs/scan; 200 TOF scans were taken per IM spectrum providing a drift time range of 0−9 ms.

RESULTS AND DISCUSSION

The aqueous derivatization of urinary creatinine by acetic anhydride was initially investigated off-line using urine spiked

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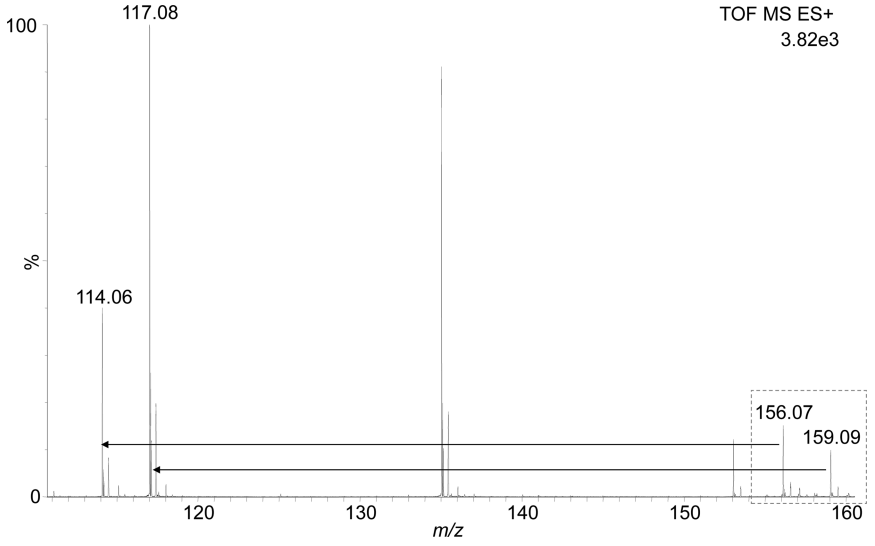

Figure 2. ESI-MS/MS spectrum showing the CID fragmentation of the $[M + H]^+$ ions of monoacylated creatinine and creatinine-d_3 (m/z 156 and 159, respectively) post-three hour reaction generating the protonated creatinine and creatinine-d_3 product ions at m/z 114 and 117.

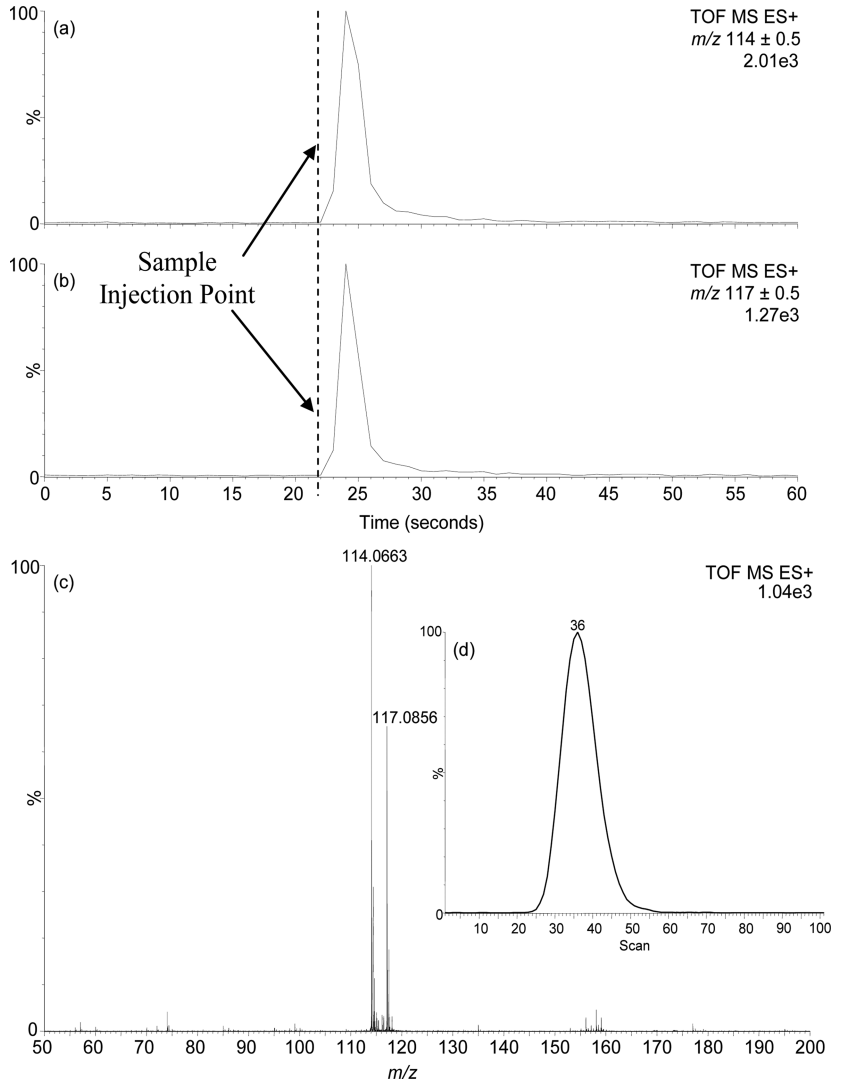

Figure 3. R-TD-EESI-IM-MS/MS selected ion responses from the analysis of urine (a) creatinine fragment (m/z 114) and (b) creatinine-d_3 fragment (m/z 117), (c) summed MS data (full width half height), and (d) IM drift profile for urinary creatinine (Bin Number 36, Drift Time = 1.6 ms).

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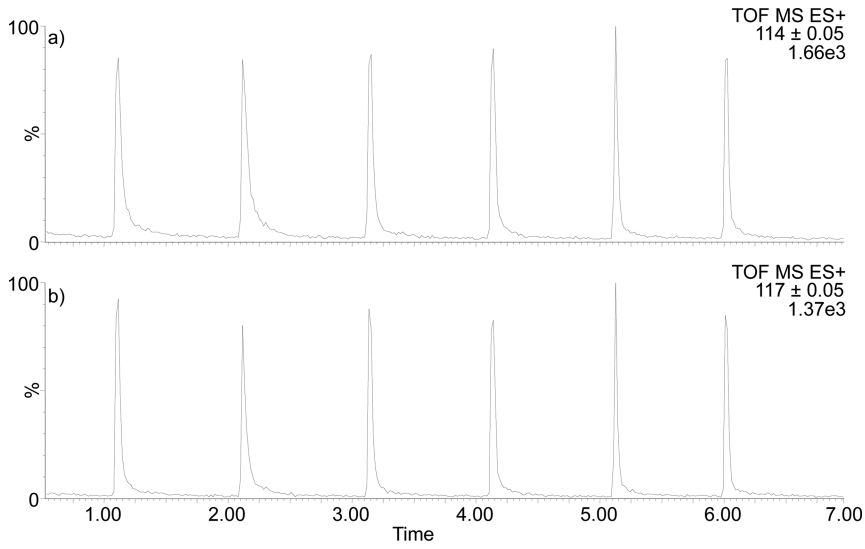

Figure 4. The selected ion responses for (a) urinary creatinine fragment (m/z 114) and (b) creatinine-d_3 fragment (m/z 117) from six replicate injections of a 1:1 mixture of urine, spiked with creatinine-d_3, and acetic anhydride using the R-TD-EESI-IM-MS/MS method.

with creatinine-d_3. The resulting solution was mixed 1:1 with acetic anhydride and allowed to react at room temperature for 3 h. Aliquots of the reaction mixture were removed, diluted, and directly infused into the ESI source of the mass spectrometer. The $[M + H]^+$ responses for the monoacylated urinary creatinine and creatinine-d_3 were observed at m/z 156 and m/z 159, respectively (Figure 2), demonstrating the derivatization of creatinine in an aqueous environment. The monoacylated ions were mass-selected and fragmented by collision-induced dissociation, resulting in the formation of fragment ions at m/z 114 and 117, which correspond to protonated creatinine and creatinine-d_3, following loss of the acetyl group, confirming the identification of the monoacyl derivatives. The direct infusion data also shows two unidentified ions present at m/z 135 and 153, associated with the urine matrix.

The direct on-probe acylation of urinary creatinine was tested under thermal desorption conditions on the TD probe to determine the volatility of the monoacyl derivative. An aliquot of diluted urine was mixed with acetic anhydride (1:1) and immediately injected into the preheated (100 °C) TD probe sample holder. The vapor plume generated by TD was contained by the stainless steel tube interface (Figure 1) and ionized by interaction of the vaporized analytes with the electrospray droplets, a process that has been termed as extractive electrospray.[25]

The direct reactive derivatization-thermal desorption (R-TD) of urinary creatinine in the presence of acetic anhydride generates a near instantaneous response for the monoacylcreatinine ($[M + H]^+$ m/z 156) and the protonated creatinine fragment ion (m/z 114). Injection of an aliquot of urine without acetic anhydride resulted in a small baseline response with a peak area <0.4% of the response in the presence of the derivatizing agent. These results show that the acylation significantly increases the volatility of creatinine from a decomposition point of ∼300 °C to less than the TD probe temperature of 100 °C and that the reaction is sufficiently rapid to allow direct on-probe derivatization and thermal desorption. Since only volatile molecules and derivatives capable of being thermally desorbed at 100 °C will interact with the ESI plume, ion suppression by the urine matrix, observed when diluted urine is directly infused into an ESI ion source,[10] is avoided by this R-TD ambient ionization approach.

Selectivity in the determination of creatinine was achieved preionization by R-TD and postionization by IM and tandem mass spectrometry to reduce chemical noise. Typical data obtained for the analysis of urine containing creatinine and creatinine-d_3 incorporating an ion mobility-tandem mass spectrometry separation after mass selection of the monoacylated ions are shown in Figure 3.

The selected ion responses for the protonated creatinine (m/z 114) and creatinine-d_3 (m/z 117) fragment ions (Figure 3a,b), from the R-TD-EESI-IM-MS/MS analysis of the mass-selected monoacylated derivatives, show the effective fragmentation of the acyl derivatives at m/z 156 and m/z 159. The mass spectrum obtained by averaging spectra across the width of the selected ion response (SIR) peak at half height (Figure 3c) shows the protonated creatinine and creatinine-d_3 fragment ions. The identification of creatinine was confirmed by accurate mass measurement of the $[M + H]^+$ ion (m/z 114.0663, mass accuracy 3.5 ppm) using creatinine-d_3 as reference standard. The inset (Figure 3d) shows the mass-selected ion mobility spectrum for m/z 114 fragment ion. The drift time of an ion is determined by the collision cross section (i.e., size and shape) of the ion as it passes through the TWIMS drift cell in the presence of a buffer gas (N_2) and under the influence of traveling wave electric field.[26,27] The drift profiles for the urinary creatinine and creatinine-d_3 have a peak maxima corresponding to a drift time of 1.62 ms, that matches the drift time obtained from creatinine standards. The time for the ion mobility separation is short (9 ms) compared to the creatinine desorption profile time (peak width at half height ∼3 s) allowing the acquisition of sufficient data points across the desorption profile. The combination of high resolution tandem mass spectrometry and ion mobility separation provides selectivity that supports the use of the direct derivatization and ambient ionization without prior chromatographic separation for the rapid determination of urinary creatinine.

The quantitative response for creatinine standards, spiked with creatinine-d_3 (5 μg/mL), determined by R-TD-EESI-IM-MS/MS was linear in the range of 2.5−20 μg/mL (R^2 = 0.986) based on the peak area ratios from six replicate injections for

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each point in the calibration curve. This corresponds to endogenous creatinine levels of 0.25−2 mg/mL in urine with the dilution taken into account. The limit of detection was 0.4 μg/mL (signal/noise, 3:1), and the limit of quantitation was determined to be 1.35 μg/mL (signal/noise, 10:1).

The potential for the rapid, high throughput determination of urinary creatinine was evaluated using replicate aliquots of diluted urine, mixed with acetic anhydride prior to injection at one minute intervals and analysis by R-TD-EESI-IM-MS/MS. Figure 4 shows the SIR responses for the urinary creatinine (m/z 114) and creatinine-d_3 (m/z 117) fragments ions in the drift time region of 1.58−1.67 ms, obtained from the analysis. The thermal desorption process generates sharp desorption profiles with high intensity and peak widths of ∼3 s. The peaks show some tailing, but for each injection, the creatinine response returns to baseline before the next injection, demonstrating that there is no carry over between samples.

The six urine injections produced responses within the calibrated range with a mean creatinine concentration in the diluted urine of 14.42 μg/mL. The dilution factor (1:100) gives a urine concentration of 1.44 mg/mL, which is within the normal urinary creatinine range based upon the donor (healthy male, 30 years of age). The precision of the R-TD-EESI-IM-MS/MS technique was determined as 9.9% RSD from six replicate measurements of urine.

CONCLUSIONS

The determination of urinary creatinine has been performed by a direct on-probe aqueous acylation prior to thermal desorption and extractive electrospray-ion mobility-mass spectrometry. The rapid acylation of creatinine to its monoacyl derivative increases the volatility of the molecule enabling rapid thermal desorption. The selective determination of creatinine was achieved preionization by derivatization and thermal desorption and postionization by ion mobility separation and tandem mass spectrometry. Quantification was carried out by the use of stable isotope dilution with creatinine-d_3 over the range of 2.5−20 μg/mL with %RSDs ranging from 8 to 10% for replicate injections. The direct ambient ionization method allowed high sample throughput, with an injection to injection time of 1 min. The derivatization-thermal desorption approach is demonstrated here for the determination of creatinine but could be applied to other involatile compounds present in complex matrices through the use of specific derivatizing agents to promote volatility and desorption of a target molecule, enabling direct quantitation while reducing background matrix interference.

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Notes

The authors declare no competing financial interest.

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