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Determination of triethylenetetramine (TETA) and its metabolites in human plasma and urine by liquid chromatography–mass spectrometry (LC–MS)☆

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Abstract

A liquid chromatography–mass spectrometry (LC–MS) method has been developed to measure triethylenetetramine (TETA) and its metabolites in human samples. We identified two metabolites of TETA, N_1-acetyltriethylenetetramine (MAT) and N_1,N_{10}-diacetyltriethylenetetramine (DAT), the latter being novel. We further developed this LC–MS method for the measurement of TETA and these metabolites in human plasma and urine in a single injection. Separation of analytes was achieved on a cyano column using 15% acetonitrile, 85% water (18 MΩ), and 0.1% heptafluorobutyric acid as the mobile phase. Simultaneous MS detection was performed at $[M+H]^+$ values of 147, 189, 231 and 245, corresponding to TETA, MAT, DAT, and N_1-acetylspermine as the internal standard, respectively. This method was successfully applied to measure TETA, MAT and DAT in plasma and urine of humans receiving oral drug treatment.

Keywords: LC–MS; Triethylenetetramine; Metabolite; Copper chelator; Diabetic heart failure

1. Introduction

Heart disease leads to death in most diabetic patients [1,2]. We recently showed that triethylenetetramine (TETA, Fig. 1), a copper chelator used for the treatment of Wilson's disease [3], can reverse diabetic heart failure in diabetes [4]. It is thought that the diabetic state leads to excessive extra-cellular copper accumulation in the heart and that the ensuing copper imbalance might lead to or cause heart failure in the diabetic context [4,5]. TETA's ability to remove excessive extracellular copper makes it a candidate treatment for diabetic cardiovascular complications and as a consequence it is now undergoing Phase II clinical trials as an experimental therapy for heart failure in diabetes.

In previous pharmacological studies of TETA treatment in Wilson's disease, the parent compound was detected and measured using high performance liquid chromatography (HPLC) methods with fluorescence or conductometric detection, because TETA does not absorb ultraviolet light. However, the conductometric method has poor sensitivity [6], while the fluorescence derivatization methods using various labeling reagents all generate complex chromatograms and moreover have been optimized to detect unchanged TETA alone [7–10]. Fluorimetric methods are also associated with challenges such as: (1) whether the analyte is fully or partially labeled; (2) whether the metabolites are labeled; (3) whether the metabolites are fully or partially labeled; and (4) whether detected peaks are separated from other known or unknown metabolites.

Abbreviations: ACS, N_1-acetylspermine; CDL, curved desolvation line; DAT, N_1,N_{10}-diacetyltriethylenetetramine; ESI, electrospray ionization; HFBA, heptafluorobutyric acid; HPLC, high-pressure liquid chromatography; IS, internal standard; LC–MS, liquid chromatography–mass spectrometry; LLOQ, lower limit of quantification; MAT, N_1-acetyltriethylenetetramine; RF, radio frequency; SIM, selected-ion monitoring; TETA, triethylenetetramine

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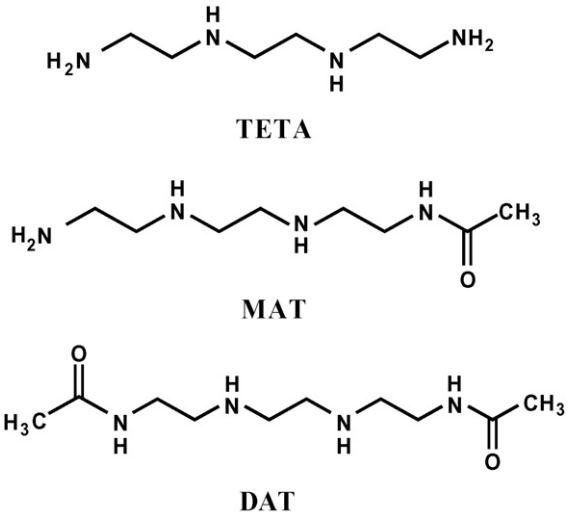

Fig. 1. Structures of triethylenetetramine (TETA), N_1-acetyltriethylenetetramine (MAT) and N_1,N_{10}-diacetyltriethylenetetramine (DAT).

Here, we report that TETA generated two major metabolites using the newly-developed liquid chromatography–mass spectrometry (LC–MS) method. One was N_1-acetyltriethylenetetramine (MAT, Fig. 1), which has previously been identified in human urine from TETA-treated subjects [11]; and the other was N_1, N_{10}-diacetyltriethylenetetramine (DAT, Fig. 1), which to our knowledge has not been reported previously. We further developed the LC–MS method to measure TETA and these two major metabolites in human plasma and urine from a single sample injection without the need for fluorescence labeling. We also report the successful measurement of concentrations of TETA and its metabolites in the plasma of one healthy volunteer and in urine samples collected from several diabetic patients after oral TETA administration as the dihydrochloride salt.

2. Experimental

2.1. Chemicals and reagents

TETA dihydrochloride (purity 99.93%), MAT trihydrochloride (98.50%) and DAT dihydrochloride (95.43%), were synthesized and supplied by CarboGen AG (Hunzenschwil, Switzerland). The internal standard N_1-acetylspermine (ACS) dihydrochloride, putrescine, cadaverine, spermidine, N_1-acetylspermidine, and heptafluorobutyric acid (HFBA) were from Sigma–Aldrich. Diacetylspermidine and N_8-acetylspermidine were a generous gift from Professor Nikolaus Seiler (Institut de Recherche contre les Cancers de l'Appareil Digestif, IRCAD, Strasburg, Cedex, France).

2.2. LC–MS

A Shimadzu LC–MS system (Shimadzu Corp., Kyoto, Japan) was utilized for analysis, which consisted of a LC-10ADvp pump, a DGU-14AM degasser, a SIL-10ADvp temperature-controlled auto injector, a CTO-10ASvp column oven and a 2010A single quadrupole mass spectrometer equipped with an electro spray ionization (ESI) interface. Fifty microlitres of samples were injected into the LC–MS and chromatographic separation achieved using a 5 μ Cyano 100 mm × 4.6 mm column (Phenomenex, maintained at 25 °C in a column oven) with guard (Phenomenex) using 15% (v/v) acetonitrile, 0.1% (v/v) HFBA (for better peak symmetry) in water (18 MΩ, Milli Q, Waters) as mobile phase (pH 1.5) with a flow rate of 0.5 mL/min. All samples were first analyzed using MS positive-ion scan mode with molecular range from 50 to 1000 Da in 1 s, to identify peaks corresponding to TETA and its metabolites. Thereafter, samples to which ACS had been added as internal standard (IS) were re-analyzed by LC–MS together with blank urine containing drug-related standards (TETA, MAT, DAT) with additional MS-positive selected-ion monitoring (SIM) signals set at $[M+H]^+$ values of 147, 189, 231 and 245 Da (corresponding to TETA, MAT, DAT and ACS, respectively) with 2 ms dwell time, in order to identify and measure the analytes. The other MS conditions were: CDL temperature 200 °C, heat block 200 °C, nebulizing gas 1.5 L/min, interface voltage 3.5 kV, CDL voltage 40.0 V, polarity positive, and Q-array voltage: DC 0.0 V, RF 140 V. The total run time was 8 min.

2.3. Detection and identification of TETA and metabolites in urine and plasma

Twenty-four-h urine samples were collected from seven patients with Type 2 diabetes immediately before and after dosing with 2400 mg of TETA dihydrochloride into pre-cleaned, wide-mouth, trace metal-free plastic receptacles (Nuplex Industries Ltd., Auckland, New Zealand) [5]. Aliquots were collected and samples were frozen and stored at −80 °C until analysis. In a separate trial, 2 mL heparinized blood samples were taken from one healthy volunteer before and at 10, 20, 40, 60, 90 and 120 min after taking 2400 mg TETA dihydrochloride. Each sample was centrifuged at 10,000 × g and plasma was collected.

For identification and measurement of TETA and metabolites, post TETA samples were thawed and transferred into 1.5 mL snap cap metal-free HPLC vials. One hundred microlitres of urine or plasma was added to 50 μL 10% HFBA and centrifuged to remove protein content especially in plasma. Supernatant (around 100 μL) was then transferred into a 1.5 mL HPLC vial with a 300 μL metal-free glass insert. The vial was then loaded onto a temperature-controlled Autosampler (Shimadzu quadrupole LC–MS 2010A). Fifty microlitres of sample was injected into the LC–MS and chromatographic separation undertaken as above with MS detection by ESI. All samples were initially analyzed in positive-ion scan mode with molecular range from 50 to 1000 Da to identify peaks corresponding to TETA and its metabolites. Thereafter, samples were re-analyzed by LC–MS together with blank urine or plasma containing TETA, MAT and DAT as well as the internal standard, with SIM settings as above and micro-scanning at 0.5 Da to improve sensitivity.

2.4. Standards and sample preparation for determination of TETA and metabolites

Samples of TETA dihydrochloride, MAT trihydrochloride and DAT dihydrochloride were dissolved in blank human plasma or urine to yield 96-μM stock solutions of each standard. Next, equal volumes of the three stock solutions were mixed (1:1:1, v/v/v) to yield a single solution containing 32 μM of each standard, which was then serially diluted in blank plasma or urine as required. Two hundred microlitres of each serial dilution was then added to 100-μL 10% HFBA and centrifuged at 12,000 × *g*. Supernatant (200 μL) was then transferred into a 1.5 mL HPLC vial with a 300 μL metal-free glass insert and 50 μL of 10 μM ACS was added as IS to final concentrations (HFBA, >50 mM; ACS, 2 μM). Human plasma and urine were processed equivalently. Samples were then analyzed by LC–MS with serially-diluted standards using the described conditions (Section 2.3) with an additional SIM signal of $[M+H]^+ = 245$ corresponding to ACS. Data acquisition and peak area automatic skim tangent integration were done using Shimadzu LCMS Solution software.

2.5. Evaluation of the method

2.5.1. Specificity and matrix effects

Ten blank plasma samples (three from the local Blood Bank and one each from seven healthy volunteers) as well as blank urine samples from six healthy volunteers and seven diabetic patients without TETA treatment [5] were analyzed using the above-described procedures. Aqueous (18 MΩ) solutions of the endogenous polyamines, putrescine, cadaverine, spermidine, spermine, N_1-acetylspermidine, N_8-acetylspermidine, diacetylspermidine and acetylspermine (Sigma–Aldrich) were also analyzed by LC–MS as described (Section 2.3). The same 13 urine and the 10 blank plasma samples to which analytes had been added, were also analyzed using the same procedure to study the matrix effects [12–14].

2.5.2. Accuracy, precision and recovery

Accuracy was evaluated by seven replicate determinations of each of TETA, MAT and DAT at four nominal concentrations (0.25, 0.5, 2 and 16 μM). Inter- and intra-batch precisions within the same day and between days were determined by analyzing seven replicates of four nominal concentrations (0.25, 2, 4 and 16 μM). The acceptance criterion was set at ±15%. Recoveries of analytes were determined in triplicate at 0.5, 1, 2, 4, 8 and 16 μM, by comparing samples to which analytes had been added before and after deproteinization. The acceptance range was 85–115%. Recovery of ACS was determined in triplicate by comparing samples to which ACS had been added before and after deproteinization, with comparison of the MS sample-peak areas corresponding to ACS. A series of serial dilutions (0.25, 1, 4 and 16 μM) was used to provide quality control (QC) samples to demonstrate dilutional linearity.

2.5.3. Calibration curves

Samples with 11 serial concentrations (0.125, 0.25, 0.5, 1, 2, 3, 4, 6, 8, 12 and 16 μM) were analyzed to establish the calibration curve. The lower limit of quantification (LLOQ) corresponding to each analyte was defined as the lowest concentration that could be reliably and reproducibly measured with concentration determinations performed in replicates of five. LLOQ criteria employed were a precision of ≤20% and a signal-to-noise ratio of ≥3.

Calibration curves were constructed by plotting analyte/IS peak-area ratios (*y*-axis) *versus* nominal concentrations of analytes (*x*-axis). The choice of regression analysis was made by selecting the curve type from amongst those available in Shimadzu LCMS Solution software whose fit yielded the highest R^2 value.

2.5.4. Stability

Tests were performed to determine the stability of three nominal concentrations (0.25, 2 and 16 μM) of TETA, MAT and DAT in each of three matrices: heparinized plasma, urine and pure water. Samples with or without HFBA were subjected to three successive 12- and 24-h freeze–thaw cycles (frozen to −80 °C), and analyte concentrations compared with freshly prepared samples in the same analytical run. Six aliquots (1 mL each) were also retained in injection vials in the LC–MS auto-sampler at 4 °C for a 54 h period, during which several injections were made to determine concentrations at specified injection times. Another set of samples with or without 50 mM HFBA were kept at room temperature, and injected into the instrument over seven successive 7-h intervals. Finally, six aliquots were added with 50 μL of concentrated HFBA, HCl, methanesulfonic acid, zinc sulfate, copper sulfate or water, respectively. We note that TETA binds strongly to both Cu^{II} and Zn^{II} ions and that these interactions may have pharmacological relevance. Samples were re-analyzed to determine whether any of these factors could modify measurements of final analyte concentrations. In a separate experiment to determine the optimal acid/concentration to stabilize analytes in biological fluids, 15 aliquots of samples containing 2 or 16 μM of each standard were also kept in the auto-sampler of the LC–MS at 4 °C for a 54 h period at the presence of 10 mM, 30 mM or 50 mM of HFBA, HCl, methanesulfonic acid, trifluoroacetic acid and nitric acid. During the 54 h period, several injections were also made to determine the concentration of analytes at the time of injection.

2.6. Measurement of TETA and metabolites in human plasma and urine samples

The suitability of the method for investigational pharmacokinetic analysis was evaluated using plasma samples from one healthy volunteer and 24 h urine samples from one diabetic patient. Urine samples contained analytes with concentrations higher than the upper limit of quantification. Therefore, these were diluted into the calibration range and HFBA was added.

3. Results and discussion

3.1. Chromatography, detection, identification, specificity, matrix effects and choice of internal standard

Before these experiments, a solution with mixed standards including TETA, polyamines and their metabolites, was injected into the LC–MS. Peaks from analytes were checked to confirm that the LC–MS system was in proper working order. Several injections were made to ensure the system was properly equilibrated. Three additional peaks were found in post-dose compared with pre-dose urine samples and the molecular weight of each determined. These were 230, 188 and 146 Da, corresponding to DAT, MAT and TETA, respectively, with confirmation of retention times and molecular masses by analysis of corresponding standards (Fig. 2). All three compounds were detected in urine samples from each diabetic subject, and all three were also detectable in the 60-, 90- and 120-min plasma samples from one healthy volunteer following 2400 mg TETA administration (Fig. 3). The analyte peaks in urine showed a second, unresolved peak on the shoulder of the analyte. The cause for this peak is unknown. It was suspected that another metal complex may dissociate back to the uncomplexed ligand in the MS source. Possible metal complexes, such as those possibly formed with Na and K, were sought by MS scanning and SIM monitoring, but were not detected. Since TETA and its metabolites are low molecular weight compounds, low concentrations of acetonitrile were used to achieve separation in a relative short run time. The final concentration of acetonitrile was set at 15%, at which asymmetric peaks were produced; therefore, ion-pairing reagent such as HFBA, nonafluoropentanoic acid or pentafluoropropionic acid were studied to optimize chromatograms. It was finally established that 0.1% of HFBA in the 15% acetonitrile mobile phase produced the best result, at which no carryover was observed. Furthermore, HFBA was also used in the samples to stabilize analytes. Therefore, it was favored to be added in the mobile phase to carry out its dual functions.

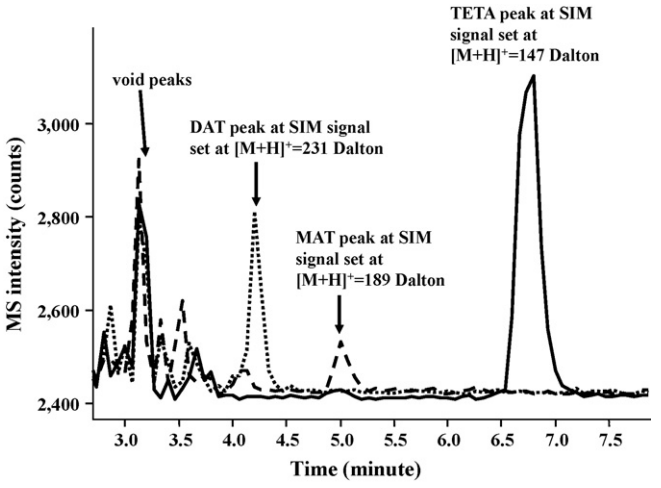

Fig. 3. MS chromatogram showing separation and detection of triethylenetetramine (TETA), N_1-acetyltriethylenetetramine (MAT) and N_1,N_{10}-diacetyltriethylenetetramine (DAT) in a plasma sample from a healthy volunteer collected at 120-min following oral TETA administration.

The endogenous polyamine, spermidine, and its metabolites, N_1 and N_8-acetylspermidine and diacetylspermidine, have molecular weights (145, 187 and 229 Da, respectively) close to those of TETA (146 Da), MAT (188 Da) and DAT (230 Da) and so have the propensity to interfere with MS measurement of TETA and its metabolites. However, using the described method, we showed that spermidine was completely separated from TETA, as was acetylspermidine from MAT and diacetylspermidine from DAT (Fig. 4).

In water, the MS peak areas corresponding to DAT, MAT and TETA at the same concentration had ratios of about 10:4:1. Biological matrices such as plasma and urine suppressed the DAT peak area significantly, suppressed MAT moderately, and enhanced TETA significantly. The peak area ratios of DAT, MAT and TETA in plasma and urine were about 5:2:2, but this phe-

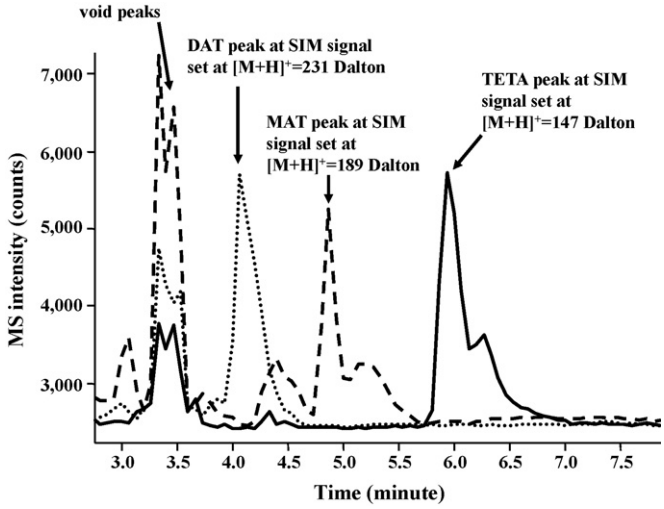

Fig. 2. MS chromatogram showing separation and detection of triethylenetetramine (TETA), N_1-acetyltriethylenetetramine (MAT) and N_1,N_{10}-diacetyltriethylenetetramine (DAT) in a urine sample from a diabetic patient following oral TETA administration (2400 mg).

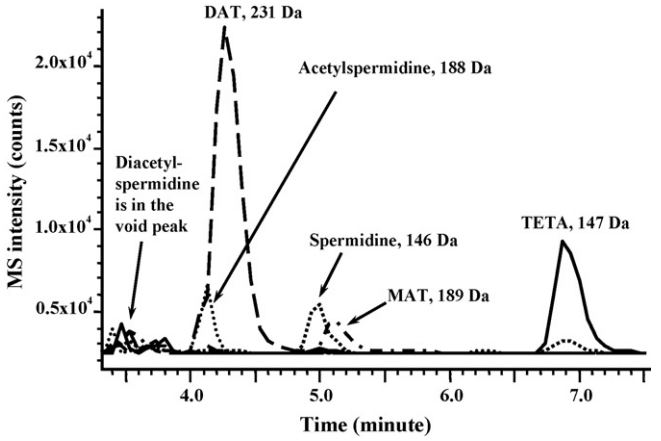

Fig. 4. MS chromatogram showing the separation of triethylenetetramine (TETA) from spermidine, N_1-acetyltriethylenetetramine (MAT) from acetylspermidine, and N_1,N_{10}-diacetyltriethylenetetramine (DAT) from diacetylspermidine.

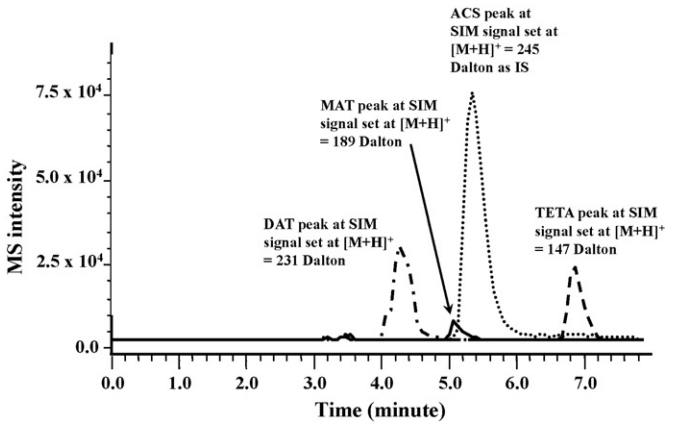

Fig. 5. MS chromatogram showing separation and detection of triethylenetetramine (TETA), N_1-acetyltriethylenetetramine (MAT) and N_1,N_{10}-diacetyltriethylenetetramine (DAT) at 4 μM each and N_1-acetylspermine (ACS, internal standard) in blank urine sample spiked with the four standards.

nomenon did not affect the LLOQ and sensitivity (calibration curve slope) of our method. Inter-individual variability in biological matrices of plasma and urine from different individuals including diabetic patients did not show variations in the MS peak areas corresponding to TETA, MAT and DAT.

The internal standard employed, ACS, is a metabolite of the endogenous polyamine, spermine. However, we did not detect ACS in any human urine samples and only two of ten plasma samples were found to contain detectable levels of ACS (both < 0.125 μM). Although ACS co-eluted with MAT, their molecular weights are substantively different (188 *versus* 244), so ACS is unlikely to interfere with the MS determination of TETA and its metabolites (Fig. 5). Co-injection of MAT and ACS showed that they did not suppress each other's ionization, with recovery rates of ACS between 96 and 101% (peak area comparison at 2 μM). Therefore, ACS is suitable for use as IS in this method.

3.2. Evaluation of the method

3.2.1. Accuracy, precision and recovery

All three analytes achieved within-run precisions in the range of 0.4–12.2% (standard deviations) in urine and 0.5–15.0% in plasma. The between-run precision (intra-day) ranged between 0.2 and 10.8% in urine and 0.1 to 15.0% in plasma. The inter-day accuracy ranged between 0.5 and 12.8% in urine and 4.1 and 10.8%. Mean recovery values at the four nominal concentrations were 90.0 ± 10.1%, 98.5 ± 16.4% and 92.6 ± 10.7% for TETA, MAT and DAT, respectively, in urine. In plasma, mean recovery values were 95.4 ± 17.0%, 92.1 ± 16.8% and 97.8 ± 9.8% for TETA, MAT and DAT, respectively. QC samples showed dilutional linearity. The dilution factor varied from 10 to 100 for urine samples and the starting concentration for urine samples varied from 30 to 600 μM.

3.2.2. Calibration curve

A line of best-fit of the analyte/IS peak area ratio *versus* analyte concentration plot was obtained using a cubic curve selected from those available in Shimadzu LCMS Solution software, as it was the only curve (compared with linear or quadratic fits) that produced a correlation coefficient (R^2) > 0.99 for each calibration curve (TETA, MAT and DAT). The range of the calibration curve was 0.25–16 μM for TETA, MAT and DAT in plasma and urine. The LLOQ was determined to be 0.25 μM, where the acceptance criteria were met (deviation 13.6%, 8.2% and 8.2% for TETA, MAT and DAT in plasma, and 15.6%, 15.4% and 7.3% in urine). Each of the remaining concentration points had a deviation <15% in the plasma and urine matrices. The linear R^2 of each regression line was >0.99 in both plasma and urine. This sensitivity for TETA determination is close to or better than those reported for previous HPLC methods. To our knowledge, no prior method existed for measurement of TETA metabolites.

3.2.3. Stability

In water, urine and plasma samples without added HFBA, three freeze–thaw cycles did not affect concentrations of MAT and DAT, and two freeze–thaw cycles did not affect TETA concentration. However, after a third freeze–thaw cycle, TETA concentrations in water, urine and plasma had deviated (decreased) from nominal concentrations. By contrast, TETA, MAT and DAT concentrations in samples with added HFBA (to 50 mM) were not affected by three freeze–thaw cycles. In injection vials at 4 °C in the LC–MS auto-sampler, TETA concentration in plasma began to deviate (decrease) from the nominal concentration after 6 h, whereas MAT and DAT concentrations were not significantly changed in the absence or presence of HFBA. The presence of ≥50 mM HFBA stabilized TETA for >36 h but deviation had begun by 42 h. At room temperature, TETA concentrations began to deviate (decrease) from the nominal concentration after 7 h, while MAT and DAT concentrations were not significantly changed in the absence of HFBA. HFBA (50 mM) stabilized TETA for 35 h but was progressively less effective thereafter. Strong acids could displace metals from TETA-metal complexes and TETA is a strong copper/zinc chelator. Therefore, strong acid was used here to release TETA from TETA-copper/zinc complexes so as to enable accurate measurement of TETA concentrations [8]. HFBA is an ion-pairing reagent used widely in chromatography, which potentially interacts with the amine groups of TETA to form a pairing-ion, thus possibly stabilizing TETA. By contrast, the other acids tested did not have any potential stabilizing effect. We added 250 mM HFBA solution to samples at a 1:4 (v/v) ratio to achieve a final concentration of 50 mM HFBA in samples. A sample volume dilution factor was taken into account in the final calculation.

Peak areas corresponding to TETA were unaltered after 24 h in aqueous solutions by addition of concentrated HFBA, HCl, methanesulfonic acid, zinc sulfate or copper sulfate. However, the addition of copper sulfate caused a significant increase in an additional peak with a molecular weight of 209 Da (Fig. 6), corresponding to a copper-TETA complex. This is considered unlikely to be an artifact occurring only at the ESI interface, as injection of the copper solution alone indicated that copper eluted much earlier, in the void peak. This phenomenon was also observed in plastic vials (data not shown).

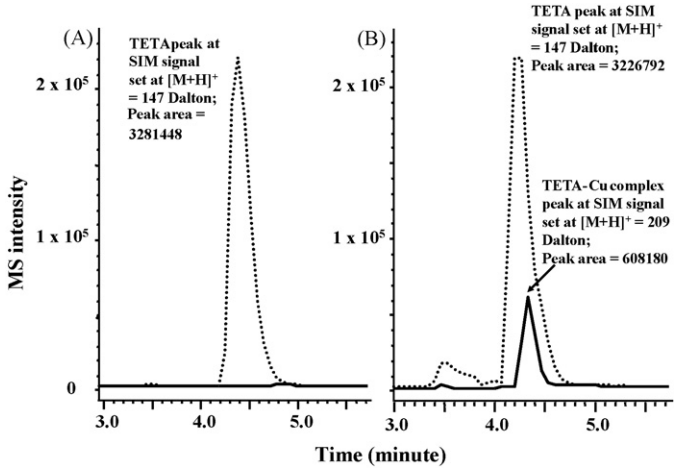

Fig. 6. MS chromatogram showing (A) the triethylenetetramine (TETA) peak in water before addition of copper sulfate, and (B) the emergence of an additional MS signal at the $[M+H]^+$ value of 209 Da, corresponding to a TETA-Cu^{II} complex, without concomitant decrease in the TETA peak area after addition of copper sulfate to the TETA solution.

Table 1
Measurement of TETA, MAT and DAT concentrations in plasma from a healthy human volunteer following a single oral dose of TETA dihydrochloride (2400 mg)

Sampling time (minutes after dose)	TETA (μM)	MAT (μM)	DAT (μM)
10	<LLOQ	Not detectable	Not detectable
20	0.69	Not detectable	Not detectable
40	5.08	<LLOQ	Not detectable
60	5.60	0.36	<LLOQ
90	10.6	1.00	<LLOQ
120	9.57	2.03	0.17

3.3. Application

This assay was applied to determine serial concentrations in plasma from a healthy volunteer, who had received a single oral dose of 2400 mg of TETA dihydrochloride (Table 1). TETA was detected in all plasma samples except the pre-treatment one, whereas MAT was only measurable in plasma samples collected 60–120 min following the dose, and DAT was measurable only in the 120-min plasma sample. Pharmacokinetic parameters were not calculated because of the limited available time points.

Table 2
Measurement of TETA, MAT and DAT concentrations in 24-h urine samples from each of seven diabetic patients collected following a single oral dose of TETA dihydrochloride (2400 mg)

Patient	TETA (μM)	MAT (μM)	DAT (μM)
1	199	254	58.3
2	44.4	244	42.7
3	71.1	525	107
4	57.2	587	178
5	30.5	333	120
6	32.7	106	37.3
7	94.4	389	64.1

The assay was also applied to measure TETA, MAT and DAT in 24 h urine samples from 7 diabetic patients after they had taken a single dose of 2400 mg of TETA dihydrochloride orally. In this case, TETA, MAT and DAT were measurable in all samples except the pre-treatment ones. The samples with analyte concentrations higher than the upper limit of quantification were diluted into the range of the standard curve. The results have been summarized in Table 2.

4. Conclusion

In summary, employing the LC–MS method described herein, we found two major TETA metabolites in human plasma and urine, one of which, DAT, has not previously been reported to our knowledge. We subsequently developed and refined an investigational LC–MS method to measure TETA, MAT and DAT concentrations in human plasma and urine samples. This method has a short run-time and is useful for determination of TETA, MAT and DAT in the relatively limited number of samples that can be processed within 36 h. However, the stability problem of underivatized TETA prevented us from extending this method to large-scale clinical analysis for pharmacology. The stability deficiencies in the present assay were due to the study starting prior to stability determinations being completed. We are currently developing another method which can label, separate and determine TETA, MAT and DAT in one injection, and which has acceptably robust performance.

Acknowledgements

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