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A sensitive method for quantitation of β-lyase metabolites of sulfur mustard as 1,1′-sulfonylbis[2-(methylthio)ethane] (SBMTE) in human urine by isotope dilution liquid chromatography–positive ion-electrospray-tandem mass spectrometry

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Abstract

A method for measurement of an important biological marker, 1,1′-sulfonylbis[2-(methylthio)ethane] (SBMTE) of sulfur mustard agent HD [bis-(2-chloroethyl)sulfide] in human urine, to quantify HD exposure, is presented. It employs $TiCl_3$ reduction of β-lyase metabolites to SBMTE, and automated solid-phase extraction sample preparation, followed by isotope dilution liquid chromatography–positive ion-electrospray ionization-tandem mass spectrometry with 7.5 min/sample cycle time, to achieve SBMTE quantitation of up to 200 samples/24 h a day. Percent relative standard deviations over the calibration range varied from 12.0% at 0.1 ng/mL to 0.9% at 100 ng/mL, and the limit of detection from a 0.5 mL sample was below the lowest level calibration standard of 0.1 ng/mL.

Keywords: Sulfur mustard; SBMTE; Metabolites; Urine; Liquid chromatography; Tandem mass spectrometry

1. Introduction

Sulfur mustard agent [bis-(2-chloroethyl)sulfide], designated HD, has been used as a war gas in WWI and in more recent Middle Eastern conflicts. It is a vesicant and biological alkylating agent, which can act via inhalational, cutaneous, and ocular routes of exposure [1]. A detailed history of its use and a discussion of its mechanisms of action are found in [1], and are summarized elsewhere [2,3]. Because of its ease of preparation and the existence of large stockpiles in several countries, it is of major concern for use by terrorists in a chemical attack against civilian populations, potentially resulting in a mass-casualty event. Rapid, sensitive, and selective analytical methods for biomonitoring to determine exposure to HD are needed. These should measure suitable marker compounds that are sufficiently persistent in the body fluids that exposure can be confirmed, if sampled within 10 days after the incident.

The seminal contributions to both the identification of the metabolic pathways of HD in humans and other mammals, as well as the initial biomonitoring assays for these metabolites have been made by the research group of Robin Black at the UK Porton Down research laboratories, and these are cited in the references to follow. The biological fate of HD has been studied in rats [4,3], and similar metabolites have been found in the body fluids of humans [5,6] exposed either deliberately or accidentally. There are two major routes for metabolites found excreted in urine. Some relevant structures are displayed in Fig. 1. Hydrolysis of HD leads to formation of thiodiglycol (TDG), which can oxidize to the sulfoxide (TDG–sulfoxide; not shown), and these can be conjugated at their –OH functionalities to form the respective glucuronides. HD may react with glutathione in blood and then undergo oxidation to the sulfone followed by β-lyase cleavage, leading to formation of 1,1′-sulfonylbis[methylthio]ethane (SBMTE), which in turn can have one or both of the S-ether linkages further oxidized, to yield 1-methylsulfinyl-2-[2-(methylthio)ethylsulfonyl]ethane

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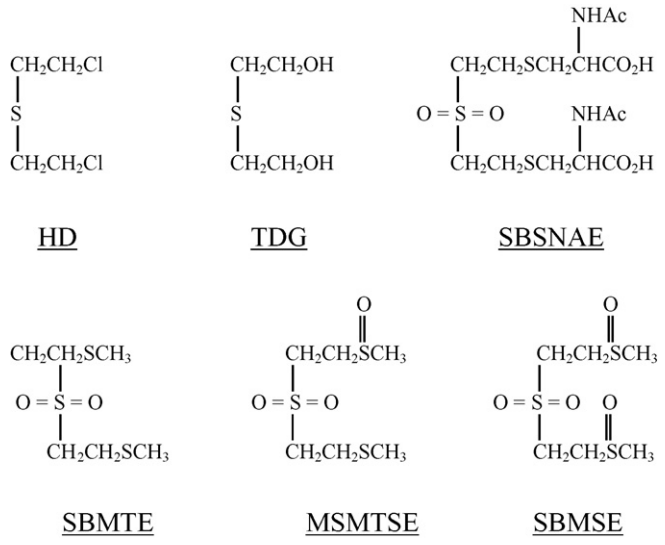

Fig. 1. Structures of sulfur mustard (HD), the primary HD hydrolysis metabolite thiodiglycol (TDG), and four HD β-lyase metabolites—SBMTE, SBMSE, MTMTESE, and SBSNAE. *Not displayed: HD and TDG–sulfoxides, sulfones, and TDG glucuronides.

(MSMTESE) and 1,1′-sulfonylbis[2-(methylsulfinyl)ethane] (SBMSE), respectively. Another glutathione-derived metabolite, 1,1′-sulfonylbis[2-*S*-(*N*-acetylcysteinyl)ethane] (SBSNAE) has also been observed [7].

HD and some of its metabolites can form adducts with DNA or protein circulating in blood or in skin [8]. These can persist for periods of weeks to possibly several months, thereby providing targets for demonstration of exposure for periods long after an exposure incident. Methods for analyzing these are complex [9–13], and are more laborious to perform.

Several analytical methods have been developed for measuring some of these HD metabolites in human urine. Wils et al. [14,15] measured TDG by converting it back to HD and measuring that by GC–MS. Black and Read [16] measured TDG in blood, plasma, and urine by GC–electron capture-negative ion-chemical ionization (NICI)-MS after derivatization, and later presented methods for determination of TDG–sulfoxide [17] and both SBMSE and MSMTESE after reduction of both to SBMTE [18] in urine by GC–MS. They later published an alternative method [20] for the latter two by LC–positive ion-electrospray ionization (ESI)-MS/MS and a method for SBSNAE [7] by LC–negative ion-ESI-MS/MS. In other work, they employed a prior $TiCl_3$ reduction treatment, to convert the sulfoxide-containing metabolites in the specific compound assays of references [17,18] to TDG and SBMTE, respectively, in a method employing deuterated internal standards [6] which thereby decreased the number and increased the levels of the HD marker compounds monitored, and improved sensitivity and selectivity by employing GC–MS/MS detection. Boyer et al. [19] used this conversion approach to develop an assay that measured both TDG and SBMTE, and they improved quantitative accuracy by incorporating ^{13}C-labeled internal standards. Their procedure yielded information on the total amounts of HD-derived material excreted in urine by each of the major metabolic paths described above, but at the cost of a laborious process requiring enzyme deconjugation, $TiCl_3$ reduction, and TDG-derivatization steps. Young et al. presented a similar but simpler method [2], which focused on measuring only the sum of β-lyase metabolites as SBMTE.

In the study by Boyer et al. [19], 80% of 105 non-HD-exposed individuals displayed urinary TDG levels after $TiCl_3$ reduction at levels between 0.5 and 20 ng/mL, but none displayed urinary SBMTE levels above the assay limit of quantitation (LOQ) of 0.1 ng/mL. Thus, SBMTE would provide a more definitive marker for HD exposure than does TDG. However, data from rat studies [3] suggest that β-lyase metabolites are cleared more rapidly than are the hydrolysis metabolites that may be quantitated as TDG. That this clearance difference may also be the case in humans was indicated by findings [5], after 2–3 days, of similar urinary SBMTE and TDG levels (∼40–80 ng/mL) in two persons suffering accidental exposure to HD, while retrospective analysis of samples collected 13 days after exposure yielded SMBTE levels of only 0.1–0.3 ng/mL, TDG levels appeared at background values [5]. Hence, if HD exposure is to be confirmed by the more specific SBMTE assay, from urine samples taken during the second week after exposure, a method with a LOQ of at least 0.1 ng/mL may be required.

We present a method for determination of SBMTE in human urine that is based on the materials used by Young et al. [2], and that employs for detection LC–positive ion-ESI-MS/MS in place of GC–positive ion-isobutane CI-MS/MS. Use of an HPLC column under isocratic conditions, instead of a long capillary GC column employing a temperature program enables attainment of shorter chromatographic sample cycle times. By automating and optimizing several of the sample cleanup steps, the method provides sufficiently clean concentrated samples to the LC–MS/MS instrument, at a rate comparable to the rate at which it quantifies them. Use of fast, automated sample preparation steps, employing two separate solid-phase extraction (SPE) analyte isolation steps, provides samples that are sufficiently clean that the desired 0.1 ng/mL LOQ can be achieved. The combination of extra sample purification from the urine matrix and the use of an atmospheric pressure ESI LC–MS/MS ion source enables large numbers of samples to be run without the need for frequent recalibration or source cleaning. The method is thus well suited for the purpose of biomonitoring HD exposure in the event of a mass casualty terrorist incident characterized by high sample loads and intense time constraints.

2. Experimental

2.1. Materials

2.1.1. Chemicals

All reagents were of analytical grade. HPLC-grade methanol, water, and dichloromethane were purchased from Burdick & Jackson (Muskegon, MI), and acetic acid and HPLC-grade acetonitrile were purchased from J.T. Baker (Phillipsburg, NJ). Sealed ampoules of $TiCl_3$ in 30% HCl, mass spectrometric purissima grade ammonium formate, and KOH were purchased from Sigma–Aldrich (Milwaukee, WI). The analytical standards and quality control (QC) materials in pooled urine and

the $^{13}C_4$-labeled SBMTE (labeled on the two ethylene moieties, synthesized at Los Alamos National Laboratory) internal standard solutions in methanol were purchased from Protocol Analytical (Metuchen, NJ), which is under contract to supply these to the Centers for Disease Control (CDC) Laboratory Response Network (LRN) participating laboratories.

2.1.2. Standards and QC solutions

The calibration standards, blanks, and QC materials were prepared and characterized by the CDC (Chamblee, GA) as described [2] and were made available through Protocol Analytical (Metuchen, NJ). All were in pooled, SBMTE-free, interference-free urine supplied by CDC. The standards were spiked with SBMTE at levels of 0.1, 0.25, 0.5, 1, 5, 20, 50, and 100 ng/mL so as to provide a 1000-fold linear calibration curve range. The two QC materials were spiked and certified as QC-low, at 2.5 ng/mL, and QC-high, at 25 ng/mL. Stability studies of these materials at CDC [16] showed that SBMTE levels measured from samples stored frozen at −70 °C for 6 months remained stable for SBMTE equivalents, measured after $TiCl_3$ reduction of any oxidized forms, as performed by their analytical methods.

2.2. Sample preparation

Aliquots of 0.50 mL of standards, QCs, blanks, and unknown urine specimens were pipetted into 15-mL screw-cap culture tubes, and 50 μL of $^{13}C_4$-labeled SBMTE internal standard solution (∼250 ng/mL) was added to each tube, followed by 1 mL of the $TiCl_3$/30% HCl reagent (ampoules were opened fresh daily). The tubes were capped and vortexed to mix, and were immersed in a 75 °C water bath for 1 h. They were removed, cooled for 5 min, opened, and 2 mL of 1N KOH solution was added to each to destroy excess $TiCl_3$ and the tubes were vortexed to mix.

Each tube was placed into 1 of 10 stations of a Caliper/Zymark RapidTrace automated SPE sample preparation apparatus, and a labeled 16 × 100 mL glass culture tube for elution collection was placed opposite it in the RapidTrace racks. Initial cleanup was on 200 mg, 3 mL Strata C18-E SPE cartridges (Phenomenex, Torrance, CA), which were conditioned with 3 mL each of methanol and water. A total of 3.5 mL was loaded from each sample tube, the SPE cartridge was rinsed with 2 mL water, and dried under positive pressure for 1 min. The cartridges were then eluted four successive times with 1 mL dichloromethane at 2 mL/min, and the combined collections in the tubes were taken nearly to dryness.

Evaporation to dryness was carried out in a vacuum evaporator (Labconco RapidVap, Kansas City, MO) operated with vortexing at 40 °C and 200 mbar pressure for 8 min.

The dried samples were reconstituted in 500 μL of dichloromethane and each tube was placed into 1 of 10 stations of a Caliper/Zymark RapidTrace automated SPE sample preparation apparatus, and a labeled 16 × 100 mL glass culture tube for elution collection was placed opposite it in the RapidTrace racks. This second stage cleanup was on 500 mg, 3 mL Strata NH_2 SPE cartridges (Phenomenex), which were conditioned with two washes of 3 mL dichloromethane, followed by loading of all the reconstituted sample volumes, and elution twice with 1 mL volumes of dichloromethane at 2 mL/min. The combined collections in the tubes were then taken to dryness.

Evaporation to dryness was under the same conditions described above, but took place in 6 min. Each dried sample was reconstituted in 150 μL of water, vortexed, and then transferred to LC autosampler vials for injection on the LC–ESI-MS/MS instrument.

2.3. LC–ESI-MS/MS analysis

Liquid chromatography (LC)–positive ion-electrospray interfaced (ESI)-tandem mass spectrometry (MS/MS) was performed on an Applied Biosystems (Foster City, CA) API 4000 instrument interfaced to an Agilent (Palo Alto, CA) 1100 liquid chromatograph equipped with a G1313A autosampler. Separations were performed on a 150 mm × 2.0 mm, 5 μm Luna C18(2) HPLC column equipped with a 4 mm × 2.0 mm Luna C18(2) precolumn, both from Phenomenex. The isocratic mobile phase was 60:40 water (made to 2 mM ammonium formate concentration):methanol, with each component containing 0.1% acetic acid. Sample injections of 15 μL were eluted isocratically at 0.5 mL/min for 6 min, with SBMTE eluting at ∼3.2 min. The next sample injection was made after approximately 1.5 min, leading to a 7.5 min analysis cycle time.

The API 4000 TurboSpray® ion source and the triple quadrupole analyzer were operated according to the following parameters: positive polarity; curtain gas and collision gas, nitrogen @ 25 and 10 psi, respectively; ion source gases 1 and 2, air @ 40 and 50 psi, respectively; ion spray voltage, 5000 V; source temperature, 450 °C; declustering, entrance, and collision cell exit potentials, @ 34, 10, and 15 V, respectively; and collision energy, 25 V. Parameters were optimized by measurement of responses at the target multiple-reaction monitoring (MRM) masses while the parameters were individually varied during a continuous infusion of the stock internal standard solution. Gases were supplied to the API 4000 instrument by a Model NM207A gas generator from PEAK Scientific, Ltd. (Chicago, IL). Mass analyses were by MRM for *m/z* 232 (MNH_4^+) → *m/z* 75 ($CH_3SCH_2CH_2^+$) for SBMTE quantitation, *m/z* 215 (MH^+) → *m/z* 75 for SBMTE confirmation, and *m/z* (MNH_4^+) 236 → *m/z* 77 for $^{13}C_4$-labeled SBMTE I.S. transitions, alternating 150 ms dwell times for each. MNH_4^+ was chosen as the primary quantitation ion, while MH^+ was reserved for confirmation use as its MRM transition to *m/z* 77 was suspected to suffer interferences at low analyte levels. The mass axis was calibrated against polypropylene glycol according to manufacturer's instructions. Representative MRM chromatograms for SMBTE quantitation and confirmation and $^{13}C_4$ SBMTE internal standard from a 0.1 ng/mL calibration standard run are displayed in Fig. 2.

2.4. Data processing and quantitation

Data were processed using Analyst® software (Applied Biosystems) supplied with the ABI 4000 instrument. All data were checked for peak selection, resolution from interferences,

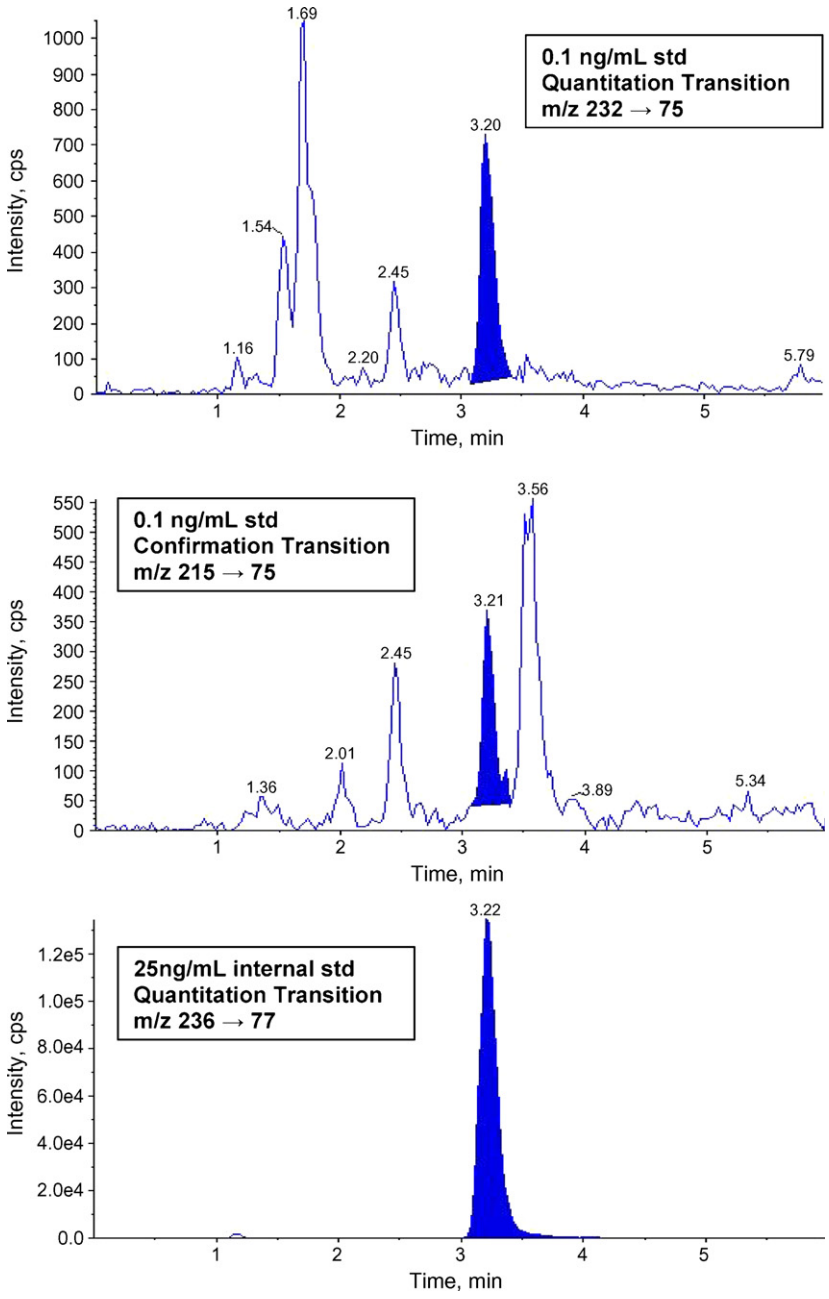

Fig. 2. LC–positive ion-electrospray interface-tandem mass spectrometric MRM quantitation and confirmation transition chromatograms of SBMTE and $^{13}C_4$ SBMTE internal standard from 15 μL injections of 0.1 ng/mL SBMTE urine calibration standard extract.

and baseline start and stop location setting, and were corrected if found to be in error. Unknown sample concentrations (i.e. QC levels and individual calibration level standards treated as unknowns) were manually integrated with the calculated value remaining blind to the operator. These were automatically quantitated using the slope and intercept from a linear regression analysis of the calibration plot data. Quality control plots were calculated through manual entry of QC data into a dedicated Excel spreadsheet program provided to the LRN labs by CDC. Other statistics were obtained through processing of the validation standard curve concentrations in Excel® (Microsoft Corp., Redmond, WA).

2.5. Human samples

One hundred different human urine samples were randomly selected from an archive which had been stored frozen at the Wadsworth Center Laboratory at −70 °C for over a year. These samples contained no identifying information as to source or gender, and no information was available to link them to any individual. The use of these urine samples was assigned an IRB exemption as Study No. 05-063 by the New York State Department of Health Institutional Review Board for use in the reference ranges study reported in Section 3.

3. Results and discussion

3.1. Method validation

Twenty validation runs each composed of eight calibration standards, a blank, and the high and low QC levels were made over a period of 13 days, with no more than two runs per day. Three additional sets of 45, 20, and 35 unknown blank urine samples were run and quantitated against validation calibration curves in the middle of the validation runs in order to acquire reference range information on 100 randomly selected blind human urine samples. The 20 calibration standard curves displayed *r*-squared values for their linear least squares fits ranging from 0.9998 to 1.0000. As summarized in Table 1, the values calculated from each of the 20 validation standard curve levels had average errors of 5.8% at the next lowest standard concentration of 0.25 ng/mL and average errors of less than 3% at 0.1 ng/mL and over the remaining 200-fold standard curve range. The mean percent relative standard deviations (%R.S.D.s) ranged from 0.9% at 100 ng/mL SBMTE to 12% at 0.1 ng/mL SBMTE. The American Chemical Society's recommendation for estimating the method limit of detection (LOD) and limit of quantitation (LOQ) as $3s_0$ and $10s_0$, respectively, where s_0 is the value of "the standard deviation as the concentration approaches zero", was employed as done in the GC–MS/MS paper of reference [2]. By taking for s_0 the *y*-intercept of the linear regression of the three lowest standards' mean deviations plotted against concentration [21], we calculated a method LOD of 0.02 ng/mL and a LOQ of 0.08 ng/mL from the data in Table 2.

Method recoveries were determined by a procedure identical to that used in reference [2]. Ratios of response factors were calculated as averages of triplicate comparisons of urines spiked with labeled SBMTE I.S. before the $TiCl_3$ reduction step (recovery) to those spiked before HPLC injection step (control) at concentrations of 0.5, 15, and 50 ng/mL. The recovery efficiencies expressed as a percent recovery ranged from 83 to 86%. These values compare favorably with the values of 18–38% obtained for the same set of analyte levels by the procedure of reference [2]. Employment of an isotope dilution internal standard introduced at the beginning of the sample preparation helps to compensate for these recovery variations.

The day-to-day precision of the calibration standards was evaluated through calculation of the %R.S.D. of the calculated concentrations of the QC urines ($n = 20$) at the high and low QC levels. Accuracy was evaluated in terms of a linear regression analysis plot of the response factor against the expected concentration. The mean %R.S.D.s were 1.7 and 1.8%, and the mean relative recoveries were 99.8 and 95.1%, for QCL and QCH, respectively, indicating the excellent day-to-day precision and relative recovery of the method.

Fig. 3 displays standard QC charts for SBMTE at the high (25 ng/mL) and low (2.5 ng/mL) levels. Each data point represents the result of a single analysis of each of the QC urines analyzed 20 times over a 2-week period. Only one QCL %R.S.D. value of 3.8% exceeded the 95% confidence intervals about the means, reflecting the excellent run-to-run precision over this period. It should be noted that analysis of very similar QC materials 6 months after initial characterization [2] produced

Table 1
SBMTE validation standard curve statistics LC–ESI-MS/MS method (means, for $n = 20$)

SBMTE, taken (ng/mL)	SBMTE, found (ng/mL)	±1S.D.	%R.S.D.	%Error	232 → 75, 215 → 75, ratio
0.10	0.098	0.012	12.0	−2.5	2.4 ± 0.3
0.25	0.236	0.014	6.1	−5.8	3.2 ± 0.3
0.50	0.491	0.026	5.3	−1.9	4.0 ± 0.5
1.00	1.02	0.04	3.4	2.3	4.5 ± 0.2
5.00	5.10	0.13	2.6	1.9	4.9 ± 0.5
20.00	20.48	0.49	2.4	2.4	5.1 ± 0.5
50.00	49.87	1.73	3.5	−0.3	5.1 ± 0.4
100.00	99.52	0.85	0.9	−0.5	5.0 ± 0.4

Table 2
Comparison of five methods for MS/MS measurement of urinary sulfur mustard metabolites

Reference	Analytes	Sample preparation	Method; instrument	I.S.	Linear range (ppb)	Cycle time (min)
This work	SBMTE	$TiCl_3$/HCl/heat, C18 SPE and NH_2 SPE—Auto RapidTrace	LC–(+)-ESI–MS/MS; API 4000	$^{13}C_4$	0.1–100	7.5
[19]	SBMTE	$TiCl_3$/HCl/heat, centrifugation, ChemElut SPE—manual	GC–(+)-isobutene CI-MS/MS; TSQ 7000	$^{13}C_4$	0.1–00	∼12
[2]	SBMTE, TDG	β-Glucuronidase, overnight, $TiCl_3$/HCl/heat, centrifugation, Oasis HLB SPE—Auto RapidTrace, HFBA derivatization	GC–(+)-isobutene CI-MS/MS; TSQ 7000	$^{13}C_4$	0.5–00	∼13
[20]	SBMSE, MSMTESE	ENV + polymericSPE—manual	LC–(+)-ESI-MS/MS; TSQ Quantum	D_6	0.1–100	∼20
[7]	SBSNAE	Oasis HLB SPE—manual	LC–(−)-ESI-MS/MS; TSQ 700	None	1–20	∼15

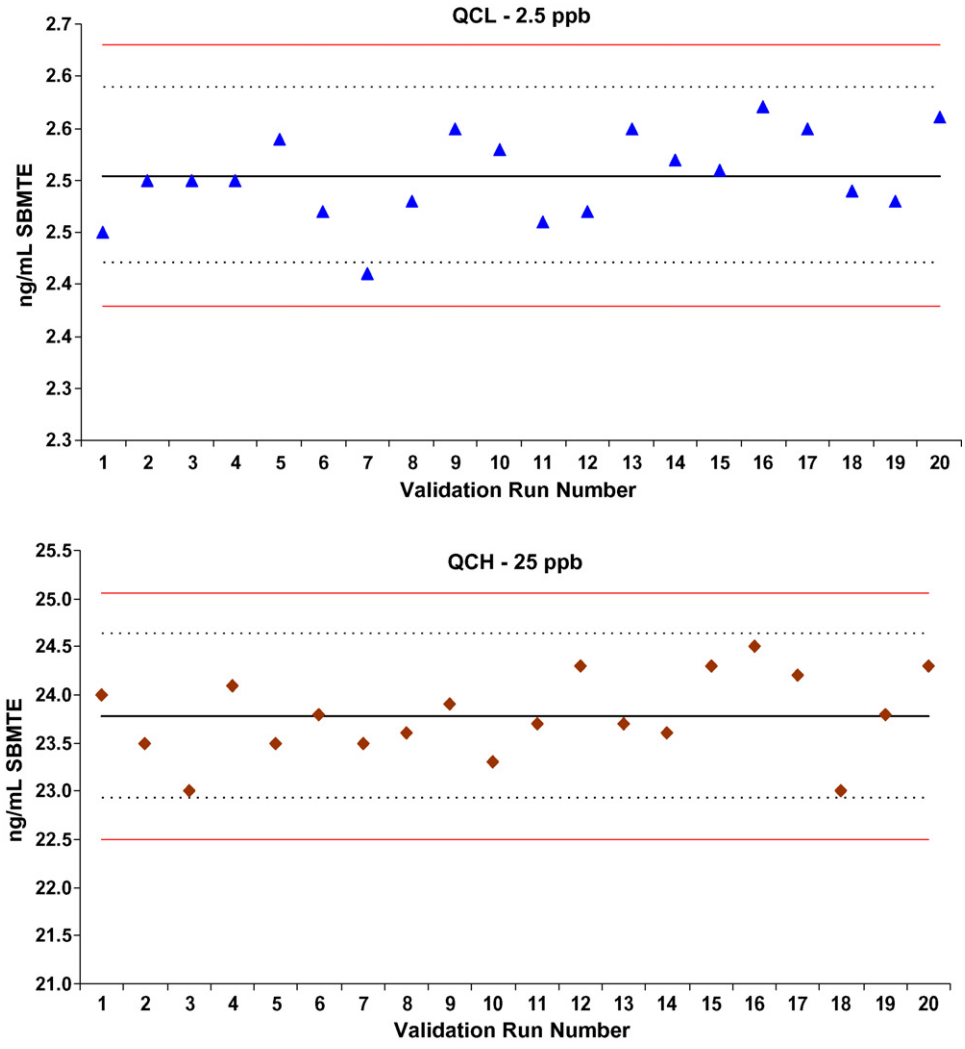

Fig. 3. Quality control charts for SBMTE in urine from LC–ESI-MS/MS method validation study at levels of 2.5 and 25 ng/mL. (- - -) 95% confidence interval; (—) 99% confidence interval.

values within the 95% confidence intervals established in that study. Storage and heat stability studies carried out on those materials [2] indicated stability at concentrations of 1, 5, and 20 ng/mL for 1 week at −70 °C, 4 °C, and ambient temperature, and urines with SBMTE concentrations of 1 and 20 ng/mL subjected to sterilization at 90 °C for 1 h showed no degradation of SBMTE nor any negative effect on the analysis. Note also that the $TiCl_3$ reduction step in the procedure would have converted any SBMSE and MTMTESE formed by oxidation of SBMTE under these conditions, back to the original SBMTE.

3.2. Reference range

The 100 human urine samples described (under "human samples") in Section 2, were analyzed in three runs carried out in the middle of the sequence of validation runs. The chromatograms from 99 of these displayed no interfering peaks at the retention time of SBMTE; i.e. peaks which could be integrated to produce a level above the LOD of 0.02 ng/mL. One displayed a peak which would quantitate at 0.06 ng/mL, which is lower than the 0.08 ng/mL LOQ and the lowest calibrator at 0.10 ng/mL. Additionally, there were no other nearby peaks that would otherwise interfere with accurate measurement of low SBMTE levels. By contrast, peaks at the retention time of SBMTE from the confirmation transition of m/z 215 → 75 were observed at magnitudes corresponding to an average concentration of 0.11 ± 0.15 ng/mL in the 100 reference range urine samples, with 7 of these ranging from 0.3 to 0.8 ng/mL. The presence of such background signals at these low levels in the confirmation transition would act to significantly increase response for that transition at the retention time of SBMTE at the lower range of calibration concentrations from 0.1 to 1 ng/mL, and correspondingly decrease the confirmation ratios at these lower analyte concentrations. Just such a lowering effect is observed in the confirmation ratios displayed in the last column of Table 1. For this reason, the NH_4^+ adduct transition instead of the MH^+ transition was chosen for quantitation.

3.3. Discussion

Young et al. [2] described the need for a more rapid, selective, and sensitive assay for SBMTE in human urine samples, to serve as a biomonitoring method for demonstrating exposure

to HD from large numbers of samples that would result from a mass casualty incident. Their assay design converted the β-lyase metabolites to SBMTE, a single analyte not found in the urine of subjects not exposed to HD, thereby conferring greater sensitivity. Use of isotope dilution capillary GC–MS/MS enabled good accuracy, precision, sensitivity to the ∼0.1 ng/mL levels likely to be encountered in urines collected between 1 and 2 weeks after significant exposure, and selectivity from urine components remaining after the simple diatomaceous earth column cleanup step that they used.

To achieve a combination of such sensitivity and selectivity, the current best practice employs isotope dilution GC or LC–tandem MS assays of different combinations of the hydrolysis metabolites (e.g. TDG; reference [19]), β-lyase metabolites converted to SBMTE [2,19], based on [6], or other individual β-lyase metabolites [20,7]. These published assays are contrasted with ours in Table 2. The critical difference highlighted in the last column of the table is the analysis cycle time for the chromatographic-MS/MS finish. The values for the procedures in the references used for comparison [2,7,19,20] are estimated from the chromatographic temperature or gradient programs described, the analyte elution times, and typical times required to reestablish initial conditions following programmed runs and for autosampler cycling.

Our initial LC–ESI-MS/MS method on the API 4000 instrument employed only a single C18-E SPE column cleanup step, and it used a 3 min isocratic LC separation on a 50 mm × 4.6 mm Onyx monolithic C18 column. Ten validation runs yielded precision, accuracy, LOD, LOQ, and QC values for the SBMTE in the Protocol urine pool matrix similar to those reported in the results section above. Application of this initial method to the 100 reference range urine samples revealed 2 flaws, however. In three instances false positive peaks indistinguishable from SBMTE at levels from 1 to 10 ng/mL were observed, and in a number of cases there were closely eluting matrix background peaks that would make difficult the accurate integration of low SBMTE level peaks. Also, as injections continued, ion suppression of the internal standard peak increased with time, and it became necessary to interrupt the run to clean off the orifice plate of the atmospheric pressure IonSpray® source. Consequently, the second NH_2 SPE column cleanup step was added, and the HPLC column length was tripled to 150 mm to improve resolution; this increased the cycle time to 7.5 min. The number of urine cleanup steps has been increased to enable use of the faster, more robust, sensitive, and selective LC–ESI-MS/MS analytical finish.

It is important to note that the fast, automated, and parallel multi-sample processing capabilities inherent in a 10-unit RapidTrace automated SPE extractor, the RapidVap multiple sample vortexing vacuum sample evaporator, and two TurboVap stations equipped with 40 tube racks functioning as a heating bath or as an alternative evaporator to the RapidVap instrument, are critical to achieving a sample preparation rate that matches the rate at which the LC–ESI-MS/MS system can process samples.

As an example, 70 unknown and duplicate (every 10th or 20th) samples plus 8 calibration, 1 blank, and 2 QC samples can be prepared in just over 5 h.

1.	60 min	Login, label tubes, pipet 0.5 mL, vortex, add I.S., add $TiCl_3$
2.	60 min	Incubate two 40-tube racks at 75 °C in water bath for 1 h
3.	30 min	Remove, let cool 5 min, add 1N KOH, vortex, load in RapidTrace trays
4.	80 min	First SPE cycle: 10 units × 8 cycles @ 10 min/cycle = 80 min
5.	20 min	Put in RapidVap for 8 min, reconstitute, vortex, and load RapidTrace
6.	40 min	Second SPE cycle: 10 units × 8 cycles @ 5 min/cycle = 40 min
7.	20 min	Put in RapidVap for 6 min, reconstitute, vortex, transfer to injection vials, cap, and load into LC–ESI-MS/MS autosampler

An estimated time of 310 min, or 5.2 h, elapses before the 80 samples (∼60 unknowns if duplicates are run every 10th sample) are ready to be measured against the calibration standards; this latter process requiring 10 h at a rate of 8 samples/h. Subsequent batches can be prepared during each ongoing LC–MS/MS run, and be measured sequentially in 10 h segments, leading to a potential sample throughput of ∼200 samples (or ∼170 unknowns)/day, assuming 24/7 operation and negligible instrument downtime. The method used for cleaning the urine samples resulted in a procedure sufficiently robust that 320 injections of 15 μL of final extract concentrates could be made over a 13-day period with no loss of sensitivity or need to clean the ion source.

4. Conclusions

We have presented an alternate method for quantitation of the β-lyase metabolites of HD in human urine as SBMTE. It achieves exceptional sensitivity, robustness, and selectivity, through multiple reaction monitoring for quantitation and confirmation transitions for SBMTE, and quantitation MRM monitoring of an isotope dilution $^{13}C_4$ SBMTE internal standard. Employment of two automated SPE cleanup steps with rapid vacuum solvent evaporation provides exceptionally clean final injection solutions at a rate compatible with continuous analysis by the fast LC–MS/MS system. Up to 200 samples and standards can be fully processed in a 24 h period. Such a capability can form the basis for the rapid determination of exposure to HD in a mass-casualty event through the analysis of large numbers of samples taken up to 2 weeks post-attack.

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