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Short communication

A sensitive high performance liquid chromatography–positive electrospray tandem mass spectrometry method for N^7-[2-[(2-hydroxyethyl)thio]-ethyl]guanine determination

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ABSTRACT

A simple, fast, sensitive and robust method for the determination of the sulfur mustard (SM) exposure biomarker–N^7-[2-[(2-hydroxyethyl)thio]-ethyl]guanine (HETEG) was reported using high-performance liquid chromatography–positive electrospray tandem mass spectrometry (HPLC–ESI-MS/MS), working in multiple reaction monitor (MRM) mode. The method provided limit of detection of 0.330 ng/mL and lower limit of quantitation of 0.940 ng/mL. The method was linearly calibrated from 0.940 ng/mL to 587 ng/mL with precisions of 3.5–14.5%, and accuracies of 88–112%. The recovery varied from 102% to 118%. HETEG spiked in DNA hydrolytes isolated from the human whole blood was stable after five freeze/thaw cycles and 35-day frozen at −20 °C. For the exposed biological samples, alkylated DNA was isolated from SM-treated human whole blood, followed by DNA digestion and adducts enrichment, the resulting alkylation base was determined. By the procedure, the HETEG level in DNA hydrolytes isolated from the human whole blood exposure to 312 ng/mL SM was detected successfully.

1. Introduction

Sulfur mustard (SM), commonly known as mustard gas (2,2′-chloroethyl sulphide), is a powerful vesicant and biological alkylating agent [1–4]. SM can react with critical nucleophilic sites in DNA and the resulting alkylated DNA can lead to miscoding, mutation and cancer eventually. Therefore, the identification and quantitation of DNA adducts is essential for evaluation of the extent of damage to the genetic material and establishment of the relationship between DNA adducts formation and other biological endpoint (mutations, DNA strand breaks etc.) [5–7]. The SM-DNA adducts can be formed in the skin, blood, and tissues of animals allegedly exposed to SM [8]. DNA nucleophilic sites of N^7-guanine, O^6-guanine and N^3-adenine have been found to react with SM and five resulting DNA adducts have been reported [9]. Among these adducts, the resulting adduct of N^7-[2-[(2-hydroxyethyl)thio]-ethyl]-guanine (HETEG) is a good biomarker of SM exposure attributed to its high abundance [9,10].

In view of the continued interest in SM, various methods including ^{32}P-postlabeling, immunoassay and chromatography coupled with different detection techniques have been reported for sensitive and accurate detection of HETEG. Early efforts to quantify HETEG involved the use of radioactively labeled SM, such as [^{35}S]-SM, after digest and subsequent separation by HPLC, the radio-labeled HETEG was quantified by virtue of the radioactive label. By this technique, both [^{35}S]-SM and [^{14}C]-SM have been used for detection of HETEG isolated from the human blood or double-strand calf thymus DNA exposure to SM [9,11]. In this kind of procedure, the synthesis of radio-labeled agent and special precautions for proper radiochemical handling are shortcomings. Immunochemical detection [12,13] and ^{32}P-postlabeling method [14] have also been used for HETEG quantitation for the high sensitivity. However, the onerous antibody and sample handling requirements in immunochemical detection systems and the false positives in ^{32}P-postlabeling method are disadvantages for the further application. In addition, attempts of using gas chromatography–mass spectrometry (GC–MS) for HETEG detection were also reported. However, the GC–MS assay failed for the poor derivation of HETEG in the presence of other bases [11]. Furthermore, there is few report of HETEG quantification by HPLC–MS approach. Only Rao et al. [15] reported the high performance liquid chromatography–electrospray mass spectrometry (HPLC–ESI-MS) method for HETEG determination providing the detection limit of 10 ng. In recent years, high performance liquid chromatography–electrospray tandem mass spectrometry

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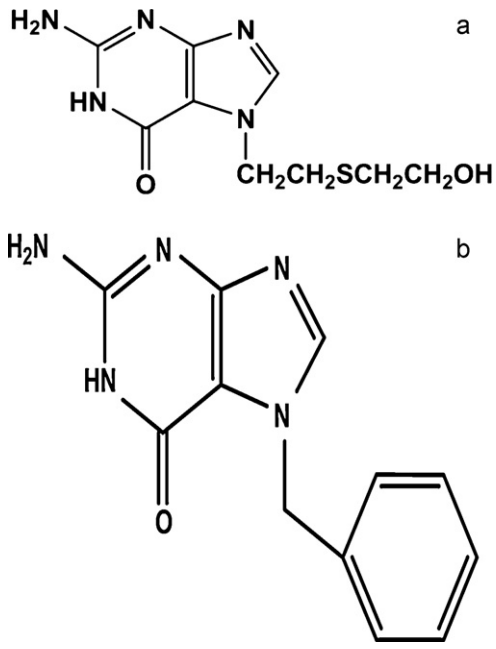

Fig. 1. Chemical structures of HETEG (a) and internal standard (b).

(HPLC–ESI-MS/MS) provided a powerful tool for DNA adducts quantitation owing to the capability of mass spectrometry to provide structural and unambiguous quantitation information in an analysis cycle [16–19].

The aim of the present study was to develop a fully validated, reliable and sensitive method for HETEG quantitation in biological matrix by HPLC–ESI-MS/MS. By this procedure, the HETEG in the SM exposed human whole blood *in vitro* was detected successfully.

2. Experimental

2.1. Caution

SM is highly reactive alkylation vesicant and cytotoxic agent. Handling of the agent by experienced personnel should be carried out in well-ventilated cupboard. Gloves and stringent protective measures should be adopted.

2.2. Chemicals and reagents

SM was provided by Institute of Chemical Defense (China). HETEG and N^7-benzylguanine hydrochloride were synthesized in our laboratory. Dichloromethane and methanol (HPLC grade) were obtained from J.T. Baker (USA). Human whole blood was supplied by healthy volunteers. Guanosine was purchased from Sigma (USA), Q-Sepharose was purchased from Pharmacia (USA). All other reagents were analytical grade, purchased from Beijing chemical works and sinopharm chemical reagents Co. Ltd (China) unless stated otherwise. Water was deionized water.

2.3. Standards

2.3.1. Synthesis of HETEG

HETEG (Fig. 1a) was synthesized based on published references with minor modifications [9]. Briefly, a solution of guanosine (GMP, 814 mg) in water (20 mL) was adjusted to pH 4.5 with 36.5 mg/mL HCl solution. SM (0.6 mL) was added, stirred for 12 h at 30 °C. The reaction mixture was neutralized with 4 mg/mL NaOH solution in order to remove the excess SM, then extracted with dichloromethane (10 mL × 3), and purified on a Q-Sepharose column. The reaction product mixture was eluted (3 mL/min) using water and 58 mg/mL NaCl solution (90 mL) in turn. The appropriate fractions were evaporated to dryness under reduced pressure and dissolved in 36.5 mg/mL HCl solution. Depurination of N^7-alkylated GMP was achieved by boiling this solution for 2 h at 100 °C. The mixture was neutralized with ammonia. The precipitate was isolated by filtration and washed by cold water. HETEG (90 mg) was obtained by vacuum drying. MS full scan (positive ion electrospray) of the resulting material gave a major protonated molecular ion with m/z = 256, the purity determined by HPLC was >96%. ^{1}H NMR (DMSO–d_6, 300 MHz) δ: 8.567 (s, lH, H-8), 6.962 (br, s, 2H, NH_2), 4.429 (t, 2H, $SCCH_2OH$), 3.543 (t, J = 6.3 Hz, 2H, NCH_2CS), 3.016 (t, J = 0.9 Hz, 2H, $NCCH_2S$), 2.607 (t, J = 6.6 Hz, 2H, SCH_2COH).

2.3.2. Synthesis of N^7-benzylguanine hydrochloride

N^7-benzylguanine hydrochloride (Fig. 1b) was used as internal standard (IS) and synthesized based on published reference [22]. Briefly, 5 g guanosine was suspended in 25 mL dimethylsulfoxide. 5 mL of benzyl bromide was added dropwise to the suspension at room temperature. Then, 12.5 mL of concentrated hydrochloric acid was added to the reaction, and the resulting reaction mixture was stirred at room temperature for 30 min. The reaction mixture was poured into 150 mL methanol, and the mixture was stirred overnight. The precipitated crystals were collected by filtration and then washed with methanol. The crystals were air-dried at 60 °C for 24 h. The resulting material gave a major protonated molecular ion with m/z 242, the purity determined by HPLC was >95%. ^{1}H NMR (DMSO–d_6, 300 MHz) δ: 8.934 (s, 1H, H-8), 7.380 (br, s, 2H, NH_2), 7.356 (m, 5H, C_6H_5), 5.510 (s, 2H, $CH_2C_6H_5$).

2.4. HPLC–ESI-MS/MS conditions

2.4.1. HPLC conditions

Analysis was performed on an Agilent ZORBAX SB C18 column (rapid resolution cartridge, 2.1 mm × 30 mm, 3.5 μm) using Agilent 1200 HPLC system (USA) incorporating binary pump, degasser, auto sampler and varied wavelength detector. Mobile phases consisted of deionized water (A) and HPLC-grade methanol (B). The gradient profile started with 5% B and linearly increased to 80% B over 8 min, then B% decreased to 5% in 2 min, and a 5-min post time was incorporated between runs. The flow rate was 0.2 mL/min. And the entire LC flow was directed into the mass spectrometer between 3.5 min and 10 min. The injection volume was 2 μL.

2.4.2. MS conditions

MS detection was executed in the positive ESI mode on an Agilent 6330 ion-trap mass spectrometer (Agilent, USA). The nebulizer was 25 psi, the dry gas was 8 L/min and dry temperature was 350 °C, respectively. Scan range is 80–260 (m/z). Quantification was performed with multiple-reaction monitoring (MRM) mode. The fragment voltage was 1 V. The precursor/product ion pairs monitored for HETEG and IS were m/z 256 → m/z 105 and m/z 242 → m/z 91, respectively.

2.5. Sample preparation

2.5.1. Exposure of human whole blood to SM

SM solutions (0.159 mg/L and 159 mg/L SM using acetonitrile as solvent) were added to 1 mL of defrosted human whole blood, and incubated for 2 h at 37 °C with continuous mixing.

2.5.2. DNA isolation

The DNA isolation was conducted according to the literature [23] with minor modification. The human whole blood were washed with 1 mL phosphate-buffered saline (PBS) by centrifugation for 15 min at 3500 × g, then the precipitant re-suspended in 1 mL lysis

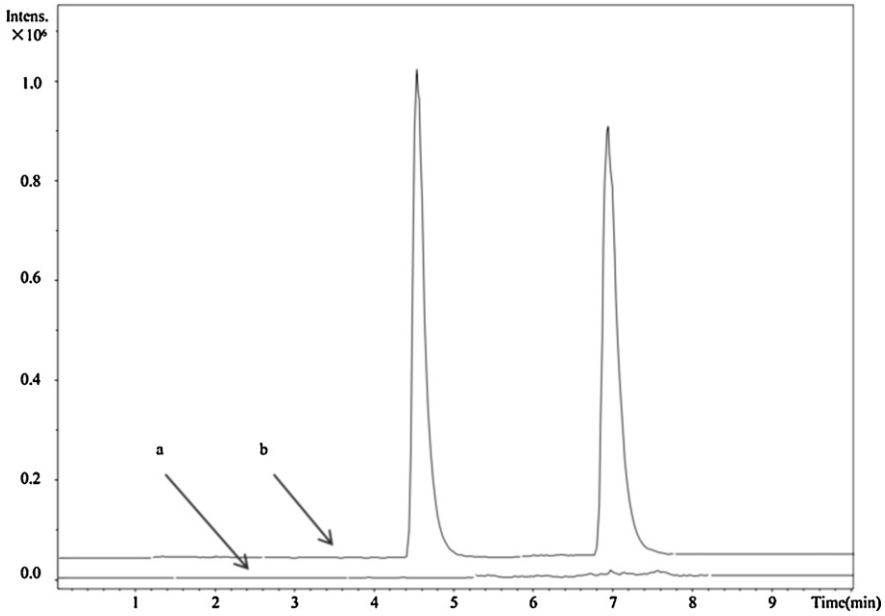

Fig. 2. No interference in the blank sample for the HETEG determination by HPLC–ESI-MS/MS in MRM mode. (a) EIC of blank run, and (b) EIC of HETEG and IS spiked sample.

buffer (1.21 mg/mL of Tris–HCl (pH 7.5), sucrose 109 mg/mL, $MgCl_2$ 475 μg/mL and Triton X-100 1%) for 30 min at 4 °C. The lysate was centrifuged at 6500 × *g* for 15 min, DNA was extracted from the precipitant using 0.5 mL extraction solution (1.21 mg/mL of Tris–HCl pH 8.3, KCl 3.72 mg/mL, $MgCl_2$ 237 μg/mL, NP-40 0.45% and Tween 20 0.45%, the RNA was removed by RNase treatment (5 μg/mL, 37 °C, 1 h), followed by proteinase K treatment (2 mg/mL, 55 °C, 3 h). The supernatant containing DNA was precipitated with cold ethanol, then centrifugation at 12,000 g for 20 min, and washed with 70% ethanol. After dryness, the DNA was re-dissolved in 0.4 mL of water and the amounts and purity of DNA were determined by UV spectrophotometry ($20A_{260\,nm}$ = 1 mg/mL DNA). The $A_{260\,nm}/A_{280\,nm}$ ratios were found to be between 1.7 and 1.9, ensuring minimal protein contamination. The yields of DNA ranged from 22 ± 8 μg/mL blood.

2.5.3. DNA digestion and adducts enrichment

The DNA was digested by acid hydrolysis. The pH of the DNA solution (0.3 mL, control and treated with SM) was adjusted to 3.5 by H_3PO_4. After mixing, the solution was heated by boiling water bath for 60 min and cooled down to room temperature. The mixture was centrifuged at 12,000 × *g* for 15 min, 0.3 mL supernatant was dried under vacuum, then re-dissolved by 70 μL water containing fixed concentration of IS (117 ng/mL) for HPLC–ESI-MS/MS analysis.

2.6. Preparation of stock, calibration standards and quality control samples

Stock solutions of HETEG and IS were prepared at 0.705 mg/mL and 2.36 mg/mL in water containing 12% formic acid (v/v), respectively. Eight calibration standards at 0.940–587 ng/mL were prepared by serial dilution by blank human whole blood DNA hydrolytes. Quality control (QC) samples at concentration levels of 0.94, 3.29,188 and 470 ng/mL were also prepared in blank human whole blood DNA hydrolytes.

2.7. Method validation

In order to demonstrate the suitability of the developed method, validation was carried out following FDA recommendations [20,21]. Linearity, intra- and inter-assay accuracy and precision, sensitivity, recovery, selectivity and stability were tested.

2.7.1. Linearity, sensitivity, precision and accuracy

The linearity was evaluated by $1/\chi^2$ weighted linear least-squares regression analysis of matrix-matched calibration curve at 0.940–587 ng/mL. The sensitivity was established by determining the lower limit of quantification (LLOQ) and the limit of detection (LOD). QC samples were used to investigate the intra-day (n = 7) and inter-day (n = 6) accuracy and precision.

2.7.2. Recovery

To determine the recovery of DNA digestion process and the quantification method, blank DNA isolated from control samples were spiked with HETEG to form the spiked samples (2.35 ng/mL, 164 ng/mL, and 470 ng/mL), then acid hydrolysis was conducted according to Section 2.5.3.

2.7.3. Stability of HETEG spiked in blank human whole blood DNA hydrolytes

The spiked samples of 2.35 ng/mL, 153 ng/mL and 470 ng/mL were subjected to five freeze/thaw cycles and frozen at −20 °C from 0 to 35 days. For each freeze/thaw cycles, the samples were frozen at −20 °C for 12 h, defrosted and kept at room temperature for 2 h.

2.7.4. Stability of SM-treated fresh human whole blood sample

Fresh human whole blood samples exposed to 145 μg/mL SM *in vitro* were subjected to seven freeze/thaw cycles and long-term freeze at −20 °C for 45 days. The exposed sample was prepared according to Section 2.5.

2.8. Assay application

The present quantification method was used to directly determine HETEG levels in a series of SM-treated human whole blood samples *in vitro*.

Table 1
Intraday and interday precision and accuracy of HETEG quality control samples.

Added concentration (ng/mL)	Intra-precision and accuracy ($n = 7$)			Inter-precision and accuracy ($n = 6$)		
	Concentration found (mean ± S.D.) (ng/mL,)	Precision[a] (%)	Accuracy[b] (%)	Concentration found (mean ± S.D.) (ng/mL,)	Precision[a] (%)	Accuracy[b] (%)
0.94	1.05 ± 0.09	8.5	112	0.96 ± 0.14	14.5	102
3.29	2.89 ± 0.11	7.5	88	3.09 ± 0.36	11.6	94
188	171 ± 6	3.5	91	180 ± 13	7.1	96
470	428 ± 16	3.8	91	479 ± 34	7.0	102

[a] Expressed as the relative standard deviation (RSD).
[b] Expressed as [(mean observed concentration/nominal concentration) × 100].

3. Results and discussion

3.1. Method development

3.1.1. Development of HPLC–ESI-MS/MS conditions for HETEG quantification

In consideration of the accuracy and reliability, internal standard method was applied. HETEG and stable internal standard (IS, N^7-benzylguanine hydrochloride) were synthesized and used for the development of HPLC–ESI-MS/MS quantification of HETEG. The chemical structure of HETEG and IS was highly similar, only different at *N*-7 substitute (Fig. 1), 2-[(2-hydroxyethyl)thio]ethyl for HETEG and benzyl for IS, respectively.

At beginning, the MS parameters of compound stability and trap drive level were optimized for the highest sensitivity by flow injection. The highest signal of HETEG and IS was achieved at the compound stability of 100% and trap drive level of 100%. Subsequently, the tandem mass spectra of HETEG and IS were investigated to choose the product ion. The protonated $[M+H]^+$ ion of HETEG (*m*/*z* 256) and IS (*m*/*z* 242) provided major product ion of *m*/*z* = 105 and *m*/*z* = 91, respectively. Therefore, the precursor/product ion pairs of *m*/*z* 256 → *m*/*z* 105 and *m*/*z* 242 → *m*/*z* 91 were used as multiple-reaction monitoring (MRM) transitions of HETEG and IS, respectively. The highest intensity was obtained when the fragment voltage was 1 V.

The stationary phase and mobile phase were also optimized for sensitivity, speed and peak shape. At first, XBridge™ C18 column (2.1 mm × 150 mm, 3.5 μm, Waters) was used for the analysis, good separation was obtained but the long analysis time of 20 min decreased the analysis throughout. Subsequently, Agilent ZORBAX SB C18 rapid resolution column (2.1 mm × 30 mm, 3.5 μm) was employed for good separation, rapid analysis and high sensitivity. Various mobile phase composition were evaluated using HPLC-grade methanol as organic solvents, and deionized water or deionized water containing formic acid (0.1%) or ammonium formate (10 mmol/L). The best sensitivity and selectivity was achieved with deionized water and HPLC-grade methanol.

3.1.2. Chromatography and selectivity

Selectivity was demonstrated by comparing blank sample and spiked sample (spiked IS and HETEG). Blank sample was analyzed for potential interferences and no interfering signals of consequence at the retention times of HETEG or IS were found (Fig. 2a). The typical extracted ion chromatogram (EIC) was shown in Fig. 2b. The overall chromatography run was completed within 10 min and post time was 5 min. Good baseline separation of HETEG and IS was observed under the optimum HPLC–ESI-MS/MS conditions and the retention times were 4.5 min for HETEG and 6.9 min for IS, respectively.

3.2. Method validation results

3.2.1. Linearity, sensitivity, precision and accuracy

The calibration curves obtained on three days was linear over the concentration ranges of 0.94–587.5 ng/mL with the correlation coefficient R^2 over 0.99. Calibration curve was $y = 0.0066x + 0.0309$. The RSD of intercept and slope were 5.6% and 3.8% over the course of the study. In the blank DNA hydrolytes isolated from the human whole blood, LOD was 0.330 ng/mL (approximately 660 fg on-column), the LLOQ of 0.940 ng/mL defined as the lowest concentration that could be measured with a precision of less than 20% (RSD). Extrapolating from the data presented previously [11], this method is capable of detecting one adduct per 9×10^8 guanines. The detection sensitivity is comparable to that of the other N^7-DNA adducts [24]. The upper quantification limit of 587.5 ng/mL was the highest concentration utilized in the matrix-matched calibration curve. The intra-day and inter-day precision and accuracy values for HETEG are shown in Table 1. The intra-day assay RSD values were less than 9% with accuracy values ranging from 88% to 112%, and the inter-day assay precision were less than 15% with accuracy values ranging from 94% to 102%.

3.2.2. Recovery

The recovery were calculated by the measured concentration to the nominal HETEG concentration before DNA digestion. And the good recovery at concentration levels of 2.35, 164 and 470 ng/mL were 118%, 116% and 102%, respectively.

3.2.3. Stability of HETEG spiked in blank human whole blood DNA hydrolytes

Freeze/thaw and long-term stability at −20 °C were analyzed against freshly prepared calibration curve and the results are

Table 2
Stability of HETEG spiked in DNA hydrolytes isolated from the human whole blood.

Added concentration (ng/mL)	Stability after 5 freeze–thaw cycles ($n = 3$)			Stability stored at −20 °C for 35days ($n = 3$)		
	Concentration found (mean ± S.D.) (ng/mL)	Precision[a] (%)	Accuracy[b] (%)	Concentration found (mean ± S.D.) (ng/mL)	Precision[a] (%)	Accuracy[b] (%)
2.35	2.84 ± 0.44	15.6	121	2.81 ± 0.41	14.6	120
153	176 ± 12	6.8	115	154 ± 18	11.8	101
470	469 ± 41	8.7	99	414 ± 23	5.6	88

[a] Expressed as the relative standard deviation (RSD).
[b] Expressed as [(mean observed concentration/nominal concentration) × 100].

Table 3
Stability of the human whole blood sample treated with 145 μg/mL SM.

Long-term stability		Freeze/thaw stability	
Days	[HETEG] (ng/mL)	Freeze–thaw cycles	[HETEG] (ng/mL)
0	5.75 ± 0.52	0	5.75 ± 0.52
2	4.62 ± 0.07	1	4.48 ± 0.80
4	5.69 ± 0.24	2	5.10 ± 0.05
6	4.51 ± 0.45	3	5.27 ± 0.52
10	5.07 ± 0.25	4	5.26 ± 0.82
13	6.41 ± 0.13	5	4.02 ± 0.06
17	5.18 ± 0.40	6	3.48 ± 0.38
27	6.44 ± 0.11	7	3.20 ± 0.48
34	5.89 ± 1.08		
45	6.25 ± 1.13		

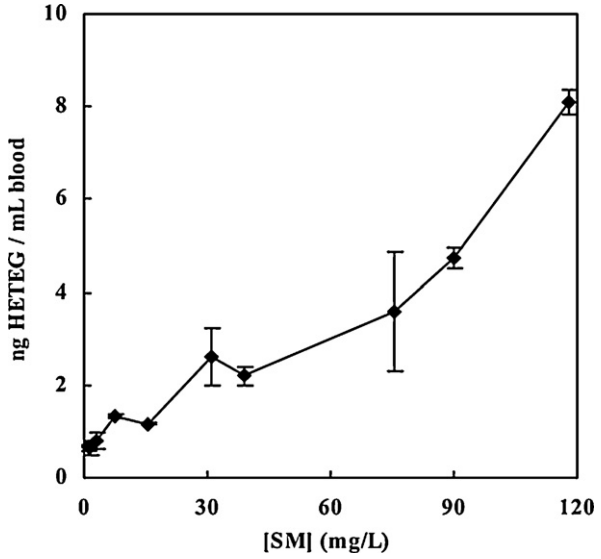

Fig. 3. HETEG levels in SM-treated human whole blood increased with the exposure dosage increase.

summarized in Table 2. Stability was evaluated by mean ± S.D., precision and accuracy. HETEG appeared to be stable in blank human blood DNA hydrolytes after five freeze/thaw cycles (mean accuracy between 99% and 121%, RSD was less than 16%) and after storage in the freezer at −20 °C for 30 days (mean accuracy between 88% and 120%, RSD was less than 15%). These results indicated that HETEG is stable after five freeze/thaw cycles and after storage at −20 °C for 30 days.

3.2.4. Stability of SM-treated human whole blood sample

Effect of freeze/thaw cycle and long term storage at −20 °C on the stability of the SM-treated fresh human whole blood sample were investigated for the discussion of the sample storage conditions and the results were listed in Table 3. It is obvious that long term storage at −20 °C had no significant influence on the detected HETEG. The amount of detected HETEG has no evident variance after 4 freeze/thaw cycles, however, the HETEG level decreased obviously from the fifth freeze–thaw cycles. The decrease of measured HETEG is due to the DNA degraded and yield decreased in the exposure blood sample that undergone more than four freeze–thaw cycles [25]. According to the results, the exposure blood sample can be stored intact at −20 °C at least 45 days prior to DNA isolation, and freeze/thaw cycle should be avoided as possible.

3.3. Quantification of HETEG in series of SM-treated human whole blood samples in vitro

The developed new assay was employed to determine HETEG in alkylated DNA extracted from a series of SM-treated human whole blood samples directly (Fig. 3). The detected HETEG level was expressed as the HETEG amount in 1 mL blood by consideration the variance of isolated alkylation DNA amount in each sample. From Fig. 3, the HETEG concentration increased with the increase of SM exposure concentration. By this procedure, the HETEG in the human whole blood sample treated with 312 ng/mL SM *in vitro* can be detected successfully, approximately 0.02 LD_{50} of rat by intravenous injection. HETEG amount in 757 ng/mL SM treated human whole blood sample can be quantified accurately. That is to say, the established procedure provided a sensitive determination technique for HETEG estimation in the real SM exposure biological sample. The method was expected to provide a means to retrospective detection and characterization the SM toxicity.

4. Conclusion

A novel HPLC–ESI-MS/MS assay for the determination of HETEG in DNA hydrolytes was established and validated completely. This method shows satisfactory sensitivity, precision, and accuracy, and it can be applied to determine the HETEG in the SM-treated human whole blood in vitro successfully. This method will provide a great deal of insight in retrospective detection, treatment assessment, carcinogenesis mechanism illustration, metabolism and genotoxicity.

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