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Determination of hexamethylene bisacetamide, an antineoplastic compound, in mouse and human plasma by LC–MS/MS

Kerri M. Smith , Wannarasmi Ketchart , Xiang Zhou , Monica M. Montano , Yan Xu

Introduction

Hexamethylene bisacetamide (HMBA) is a hybrid bipolar compound first synthesized and characterized as an erythroid differentiator for murine erythroleukemic cells (MELC) in 1976 [1]. Initially inspired by the structures and functions of DMSO and N-methylacetamide, HMBA was used as the model differentiating agent for a class compounds known as acetylated diamines. At a concentration of 5 mM, HMBA caused >99% of MELC in culture to differentiate without cytotoxicity [1]. Furthermore, experimentation showed that HMBA also induced terminal differentiation in a variety of leukemic cell lines [2,10]. Based on these findings, HMBA was studied in several phase I and II clinical trials for the treatment of myelodysplastic syndrome (MDS), acute myelogenous leukemia (AML), general advanced cancer, and solid tumors [3–9]. However, serious side effects of HMBA, such as thrombocytopenia, limited the dose escalation and prevented sufficient plasma concentrations to be realized for its terminal differentiating potential.

Recent studies show that HMBA induced the expression of an endogenous protein, hexamethylene bisacetamide inducible protein 1 (HEXIM1), which inhibits cell growth [11,12]. Increased

HEXIM1 expression in breast tumor and breast epithelial cells resulted in a decrease of cell proliferation [12]. Additionally, not only did HEXIM1 inhibit cell proliferation, it interacted with the estrogen-receptor (ER)-gene transcription complex and prevented mammary gland development *in vivo* [13]. Such properties indicated a possible new role for HMBA in the treatment of breast cancer. It has been proposed that HEXIM1 interferes with kinase action at the coding region of ER-responsive genes preventing the phosphorylation of RNA polymerase II (RNAP II) by the kinase complex, positive transcriptional elongation factor b (P-TEFb) [13,14]. Phosphorylation of RNAP II by P-TEFb facilitates complete mRNA elongation [15]. The prevention of this phosphorylation commits the cell to the abortive phase of elongation, and halts transcription [16].

To support studies of HMBA at lower doses as a potential therapeutic agent for breast cancer, a sensitive analytical method is required. Up to date, the published analytical methods for the measurement of HMBA in plasma and urine are LC–UV and GC–N/P based methods, which have lower limits of quantitation (LLOQs) of 1.00 µg/mL and 2.00 µg/mL, respectively [17,18]. These and other methods have been applied to several high-dose HMBA phase I and II clinical trials [3–9,19,20], but the LLOQs of these methods are not sufficient for the measurement of HMBA in the majority of biological samples for breast cancer study with concentrations less than 1.00 µg/mL. This paper describes, for the first time, the

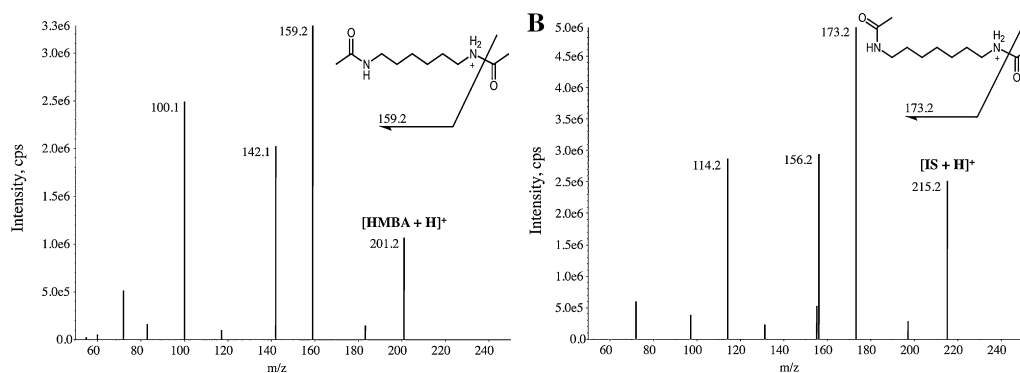


Fig. 1. Product ion spectra and structures of (A) HMBA and (B) 7MBA, the internal standard (IS).

development and validation of an LC–MS/MS method for the quantitative measurement of HMBA in both mouse and human plasma with an LLOQ of 0.500 ng/mL and a linear calibration range up to 100 ng/mL. In this work, heptamethylene bisacetamide (7MBA) was used as the internal standard. Both HMBA and 7MBA were recovered from plasma matrices by a simple step of deproteinization with acetonitrile. Separation of the analyte and internal standard was achieved on a Waters Atlantis® T3 column using 15% acetonitrile 85% 10 mM ammonium acetate, pH 4.0 as mobile phase. Quantitation was carried out by tandem mass spectrometry operated in the positive multiple-reaction-monitoring (MRM) mode. Finally, the validated method was applied to the measurement of HMBA concentrations in a preliminary mouse study.

Experimental

Chemicals and standard solutions

Ammonium acetate and hexamethylene bisacetamide (catalog no. 224235) were from Sigma–Aldrich (St. Louis, MO, USA). HPLC-grade acetonitrile was from Pharmco–AAPER (Louisville, KY, USA). HPLC-grade glacial acetic acid was from J.T. Baker through VWR (West Chester, PA, USA). Sodium chloride, sodium hydrogen phosphate, potassium dihydrogen phosphate, sodium chloride, potassium chloride, 2,2,2-tribromoethanol, and tert-amyl alcohol were from Fisher Scientific (Pittsburgh, PA, USA). Heptamethylene bisacetamide (7MBA) was obtained from the DTP Open Chemical Repository of the US National Cancer Institute (<http://dtp.cancer.gov>) with assigned code NSC36911. Six pooled blank human plasmas with specific lot numbers (W06509203366, W06509105961, W069509203227, W069509203370, W069509203365, and W069509203234) were from Haemtech, Inc (Essex Junction, Vermont, USA), which were donated by Dr. Michael Kalafatis at Cleveland State University. Six pooled blank mouse plasmas (citrated) with specified lot numbers (09F21004, 11B21080, 11B21081, 11B21082, 11B21083, and 11B21084) were purchased from Lampire Biological Laboratories (Pipersville, PA, USA). The Type 1 deionized water was obtained from a Barnstead NANOpure® water purification system (Thermo Scientific, Waltham, MA, USA).

The mobile phase for liquid chromatographic separation was prepared by mixing acetonitrile and 10 mM ammonium acetate (pH 4.0) at a ratio of 15:85 (v/v). The standard stock solutions of HMBA and 7MBA were prepared in acetonitrile at a concentration of 1 mg/mL. The standard working solutions of HMBA at concentrations 50.0, 100, 150, 500, 1.00×10^3 , 3.00×10^3 , 5.00×10^3 , 9.00×10^3 , and 10.0×10^3 ng/mL were prepared by serial dilutions of the standard stock solution of HMBA with the mobile phase. The internal standard working solution of 100 ng/mL was prepared by

two subsequent dilutions (1:100) of the standard stock solution of 7MBA in the mobile phase. The standard stock solutions were kept in amber glass vials and stored at -20°C .

Instrumentation

The liquid chromatography tandem mass spectrometry system was comprised of a AB Sciex QTrap 5500 mass spectrometer equipped with electrospray ionization (ESI) probe and syringe pump (AB Sciex, Foster City, CA, USA), and a Shimadzu Prominence UFLC system with binary pump and autosampler (Shimadzu, Columbia, MD, USA). The system was connected using PEEK tubing (1/16 in. o.d. \times 0.01 in. i.d.). Data was acquired and processed using AB Sciex Analyst software (version 1.5.1).

Liquid chromatography

Analytical separation of HMBA and the IS was performed isocratically at ambient temperature on a Waters Atlantis® T3 (3 μm , 120 \AA , 2.1 mm \times 50 mm) column (Waters, Milford, MA, USA) with the mobile phase at the flow rate of 0.150 mL/min. The injection volume of each sample was 5 μL . Prior to initial sample injection, the column was equilibrated with the mobile phase at the above flow rate for a minimum of 15 min.

Tandem mass spectrometry

The AB Sciex QTrap 5500 mass spectrometer was operated by the positive electrospray ionization (ESI) mode using the following instrument settings: CUR 34; CAD HIGH; IS 4500; TEM 550; GS1 38; GS2 32; DP 70; EP 10; CE 20; CXP 16. These settings were optimized first by direct infusion of 200 ng/mL each HMBA and the IS at 10 $\mu\text{L}/\text{min}$ using the integrated syringe pump, then refined by the “Compound Optimization” feature of the Analyst software using flow injection analysis. HMBA and the IS were quantitated by MRM mode using the following mass transitions: m/z 201.2 \rightarrow 159.2 for HMBA and m/z 215.2 \rightarrow 173.2 for 7MBA, with a dwell time of 300 ms for each analyte.

Plasma calibrators and quality controls

Plasma calibrators and quality controls (QCs) were prepared using the pooled blank human and mouse plasmas which contained no detectable HMBA. Plasma calibrators were prepared by addition of 10 μL of the mobile phase (for the blank of HMBA) or each standard working solution of HMBA (50.0, 100, 500, 1.00×10^3 , 5.00×10^3 , and 10.0×10^3 ng/mL) to 990 μL of blank pooled plasma for final concentrations of 0.00, 0.500, 1.00, 5.00, 10.0, 50.0, and 100 ng/mL each in a 1.5-mL microcentrifuge tube

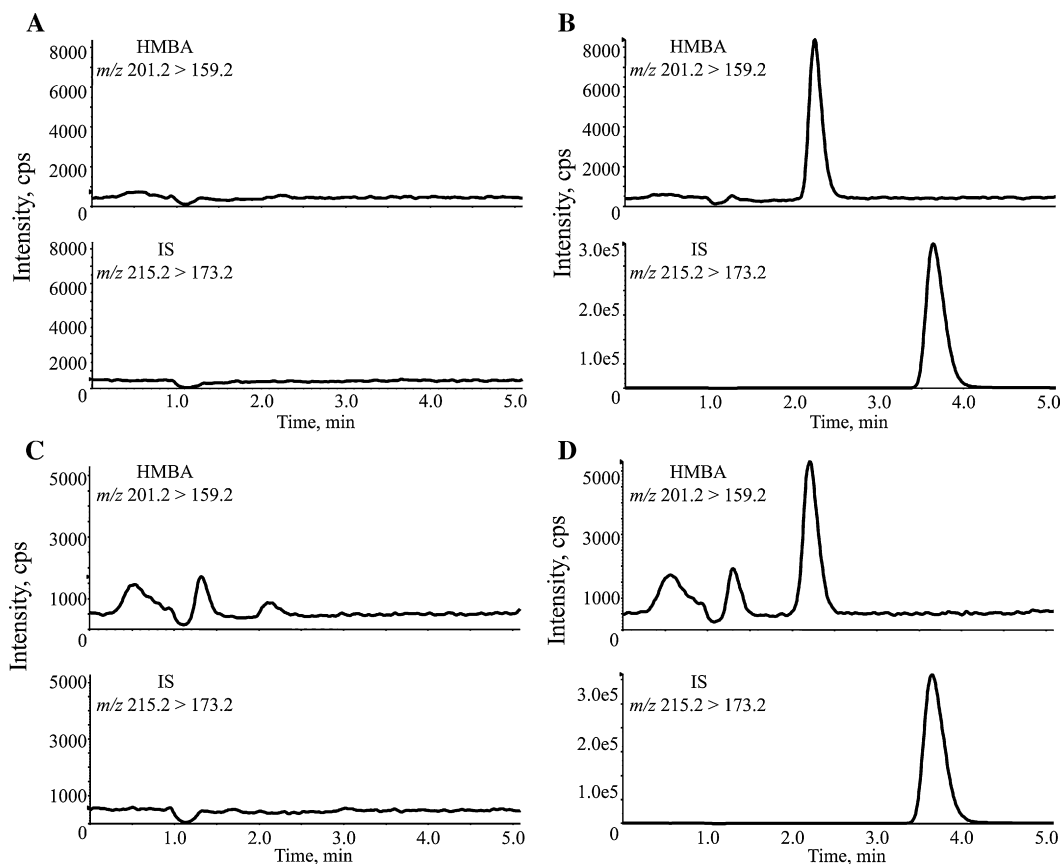


Fig. 2. Representative MRM chromatograms of human plasma: (A) double blank and (B) 0.500 ng/mL HMBA (LLOQ), 20.0 ng/mL IS and mouse plasma; (C) double blank and (D) 0.500 ng/mL HMBA, 20.0 ng/mL IS.

(VWR, West Chester, PA, USA). Plasma QCs were prepared by addition of 10 μ L of each standard working solution of HMBA (150, 3.00×10^3 and 9.00×10^3 ng/mL) to 990 μ L of blank pooled plasma for final concentrations of 1.50, 30.0, and 90.0 ng/mL each in a 1.5-mL microcentrifuge tube. The plasma calibrators and QCs were vortex-mixed for 30 s, and then stored overnight at -20°C before use.

Animal study

The animal study protocol for this work was approved by the Institutional Animal Care and Use Committee at Case Western Reserve University. FVB mice from Jackson Laboratories (Bar Harbor, ME, USA) at 4–5 weeks of age were anesthetized using Avertin (containing 1.3% tribromoethanol and 0.8% tert-amyl alcohol). HMBA (10 mg/kg) in saline (0.9% sodium chloride in water) was then injected into the mammary tissue through the nipple. At 0 (pre-dose), 15, 30, 45, 60, 120, 240, 360, and 480 min, the mice were ocularly bled and then sacrificed. Blood samples were collected in sterile 1.5-mL microcentrifuge tubes and centrifuged at $3000 \times g$ for 15 min. Mouse plasma samples were stored at -20°C until analysis. The developed LC/MS method was used to determine the concentrations of HMBA in mouse plasma over the time course of study.

Sample preparation

Plasma calibrators and QCs, as well as mouse plasma samples from FVB mice, were prepared as follows: samples were removed from -20°C freezer, and thawed to room temperature; for each plasma sample, 25 μ L of plasma together with 5 μ L of the IS work-

ing solution (100 ng/mL) or the mobile phase (for the blank of IS) were added to 1.5-mL microcentrifuge tube, vortex-mixed for 30 s, and kept at 4°C for 30 min; the sample was then deproteinized with 100 μ L of HPLC-grade acetonitrile at a ratio of 3.3 to 1 by vortex-mixing for 30 s; following centrifugation at $24,400 \times g$ for 10 min, the supernatant was pipetted into a 1.5-mL microcentrifuge tube and dried in a Savant DNA120 SpeedVac[®] concentrator (Thermo Scientific, Asheville, NC, USA) at 43°C for 30 min; finally, the resultant residual was reconstituted in 25 μ L of the mobile phase for the subsequent LC–MS/MS analysis.

Stability studies

The stability of HMBA in human and mouse plasma before and after sample preparation, and through freeze-and-thaw cycles were investigated at low, medium, and high QC concentrations. These studies included QC samples kept on bench top at 22°C for 0, 4, 8, and 24 h before sample preparation and analyses, QC samples kept in autosampler at 4°C for 0, 4, 8, and 24 h after sample preparation and before LC–MS/MS analyses, and QC samples undergone three freeze-and-thaw cycles where the samples were frozen at -20°C for at least 24 h and thawed at room temperature unassisted 3 times.

Results and discussion

Liquid chromatography

Due to its chemical structure, HMBA has a propensity to interact with the particle substrate of the bonded phase resulting in a tailing peak. Therefore, columns with endcapping (e.g., Waters

Table 1
Recovery and matrix factor of HMBA in human and mouse plasma.

	Nominal [HMBA] (ng/mL)	$A_{\text{HMBA}}/A_{\text{IS}}$ in plasma \pm SD	$A_{\text{HMBA}}/A_{\text{IS}}$ in matrix \pm SD	$A_{\text{HMBA}}/A_{\text{IS}}$ in mobile phase \pm SD	Recovery ^a \pm SD (%)	MF ^b \pm SD
Human (n=3)	1.50	0.053 \pm 0.001	0.055 \pm 0.001	0.054 \pm 0.001	96 \pm 1	1.02 \pm 0.03
	30.0	1.04 \pm 0.003	1.05 \pm 0.01	1.05 \pm 0.01	99 \pm 1	1.00 \pm 0.01
	90.0	2.92 \pm 0.01	3.01 \pm 0.02	2.95 \pm 0.02	98 \pm 0.2	1.02 \pm 0.01
Mouse (n=3)	1.50	0.050 \pm 0.001	0.049 \pm 0.001	0.051 \pm 0.002	102 \pm 1	0.96 \pm 0.03
	30.0	0.94 \pm 0.002	0.97 \pm 0.04	0.96 \pm 0.01	97 \pm 4	1.01 \pm 0.01
	90.0	2.66 \pm 0.004	2.68 \pm 0.02	2.70 \pm 0.03	99 \pm 1	0.99 \pm 0.01

^a Recovery = [(mean area ratio of HMBA to IS in plasma sample)/(mean area ratio of HMBA to IS in plasma matrix after extraction)] \times 100%.

^b Matrix factor (MF) = (mean area ratio of HMBA to IS in plasma matrix after extraction)/(mean area ratio of HMBA to IS in mobile phase).

XBridgeTM C8, Waters XTerra[®] C8, and Waters Atlantis[®] T3) were considered for analytical separation. Among the columns tested, Waters Atlantis[®] T3 (2.1 mm \times 50 mm, 3 μ m) displayed not only excellent retention times and reproducibility for the analytes, but also symmetrical peak shapes without adding additional modifiers to the mobile phase. Therefore, it was chosen for this work.

Mass spectrometric detection

Full-scan infusion analysis revealed [M+Na]⁺ as the predominant precursor ion in the aqueous solutions of both HMBA and the IS (mass spectra not shown). The addition of an ammonium salt was effective to suppress the formation of [M+Na]⁺ and produce [M+H]⁺ as the major precursor ions. After investigation with each ammonium acetate and ammonium formate, it was determined that the former resulted in greater detection signal; therefore, ammonium acetate was added to the mobile phase in the subsequent studies. Precursor ions [HMBA+H]⁺ at m/z 201.2 and [IS+H]⁺ at m/z 215.2 produced major product ions at m/z 159.2 and m/z 173.2 by breaking the amide bond (Fig. 1). Therefore, the mass transitions of m/z 201.2 \rightarrow 159.2 for HMBA and m/z 215.2 \rightarrow 173.2 for the IS were used for the quantitation of HMBA by tandem mass spectrometry with MRM mode.

Matrix interference and specificity

The use of Waters Atlantis[®] T3 (2.1 mm \times 50 mm) as analytical column for separation and 35% methanol and 5 mM ammonium acetate (pH 6.8) as mobile phase was first evaluated. While achieving excellent retention and separation for HMBA and the IS, interferences were encountered in both plasma matrices, more severely in mouse plasma (chromatograms not shown). This unidentified endogenous compound co-eluted and produced a common product ion of m/z 159.2 with HMBA in the tandem mass spectrometer.

Table 2
Accuracy and precision of plasma calibrators (n=7) over three different days.

Nominal [HMBA] (ng/mL)	Accuracy (%E) ^a		Precision (%CV) ^b	
	Human plasma	Mouse plasma	Human plasma	Mouse plasma
0.500	1	1	3	6
1.00	-1	3	2	2
5.00	-0.5	-3	3	2
10.0	2	-2	4	5
50.0	-3	2	2	3
100	1	-0.6	2	1

^a %E = [(measured [HMBA] - nominal [HMBA])/nominal [HMBA]] \times 100%.

^b %CV = (standard deviation/mean value) \times 100%.

Since choosing a different product ion for quantitation of HMBA reduced the sensitivity of detection significantly, other approaches to minimize the interference were examined: (i) various sample preparation methods, such as (a) protein precipitation using various volume ratio of plasma to organic solvent(s) [i.e., plasma to acetonitrile ratio of 1:2, 1:3, 1:4, and 1:5 with and without 0.1% formic acid or 0.1% ammonium hydroxide modifiers; plasma to acetonitrile/methanol (75/25) ratio of 1:5, as well as plasma to acetonitrile/ethanol (75/25) ratio of 1:5]; (b) liquid-liquid extraction with saturated ammonium sulfate and 15/85 isopropanol/ethyl acetate [21], and (c) denaturation of plasma proteins by heating the sample at 100 $^{\circ}$ C for 5 min prior to centrifugation; (ii) separation by different types of column [e.g., Waters XBridgeTM C8 (2.1 mm \times 50 mm, 3 μ m, 120 \AA) and Waters XTerra[®] C8 (2.1 mm \times 50 mm, 3 μ m, 120 \AA)]; and (iii) changing mobile phase composition and/or pH.

All sample preparation methods tested yielded similar results except liquid-liquid extraction with saturated ammonium sulfate and 15/85 isopropanol/ethyl acetate, which worsened the interference. Among the columns examined, the Waters Atlantis[®] T3 (2.1 mm \times 50 mm) column displayed the best chromatographic performance. The most satisfactory results were obtained by changing the mobile phase organic composition and pH (i.e., 15% acetonitrile, 85% 10 mM ammonium acetate at pH 4.0) for both human and mouse plasma.

Finally, the optimal separation of HMBA and the IS was achieved on a Waters Atlantis[®] T3 (2.1 mm \times 50 mm) column at 2.2 and 3.7 min by a mobile phase containing 15% acetonitrile, 85% 10 mM ammonium acetate at pH 4.0 using 3.3 volumes of acetonitrile for deproteinization. Under these conditions, the previously co-eluted interference was completely resolved from human plasma (Fig. 2A and B). Even though the interfering compound in mouse plasma was not completely removed (Fig. 2C, top trace), it had been reduced to a minimum that was insignificant for the analysis. The peak area of the interference in mouse plasma was about 8% of the LLOQ for HMBA by the LC-MS/MS method (Fig. 2C and D), an acceptable level by the industry [22]. The specificity of the LC-MS/MS method was further demonstrated by measuring HMBA at the LLOQ (0.500 ng/mL) of the method from six lots of human plasma samples and six lots of mouse plasma samples (see Section 3.4.2).

Method validation

The method was validated following the guidelines brought forth by the FDA's Bioanalytical Method Validation Guidance for Industry [22,23].

Recovery and matrix factor

Recovery was calculated by comparing the mean-peak-area ratios of HMBA to the IS of corresponding QC samples prepared by spiking the analytes to plasma matrix before and after plasma

Table 3
HMBA at LLOQ in different lots of human and mouse plasmas.

Nominal [HMBA] (ng/mL)		Lot A	Lot B	Lot C	Lot D	Lot E	Lot F
		0.500	0.500	0.500	0.500	0.500	0.500
Human (n = 5)	Mean	0.525	0.502	0.491	0.463	0.461	0.472
	Standard Dev (SD)	0.010	0.004	0.014	0.002	0.004	0.006
	Accuracy (%E)	5	0.3	-2	-7	-8	-6
Mouse (n = 5)	Precision (%CV)	2	1	3	0.5	1	1
	Mean	0.528	0.486	0.498	0.489	0.475	0.497
	Standard Dev (SD)	0.013	0.043	0.031	0.035	0.010	0.028
	Accuracy (%E)	6	-3	-0.3	-2	-5	-1
	Precision (%CV)	3	9	6	7	2	6

Each datum point calculated by five parallel measurements from five identical QCs.

deproteinization. Matrix factor was calculated by comparing the mean-peak-area ratios of HMBA to the IS in the QC samples prepared by spiking the analytes after plasma deproteinization to those prepared in the mobile phase. For these studies, triplicate measurements were performed for all low, medium, and high QC concentrations.

Table 1 shows that the recoveries of HMBA were consistent and between 96% and 98% in human plasma, and 97% and 102% in mouse plasma over the three concentrations examined. Deproteinization by 3.3 volumes of acetonitrile was sufficient to recover HMBA from the matrices.

Matrix factor is a measure of sample matrix effect (either suppression or enhancement) on analytical signal of HMBA. In this work, matrix factor for human and mouse plasma ranged 0.96–1.02 (Table 1), which indicated that the plasma matrix effect was in the magnitude of -4% to 2% ($\leq 15\%$). Hence, the plasma matrix suppression or enhancement of the analytical signals was no significant and could be neglected without further correction.

Calibration curve and lower limit of quantitation

The linear calibration ranges of 0.500–100 ng/mL were established for HMBA in both human and mouse plasma with internal standard using six non-zero calibrators, a single-blank (IS only), and a double-blank. The calibration equations derived from seven individual calibration curves on three different days with $1/x$ weighting were $y = 0.0287(\pm 0.0046)x + 0.084(\pm 0.0043)$, $r^2 = 1.00$ for human plasma, and $y = 0.0294(\pm 0.0030)x + 0.0118(\pm 0.0057)$, $r^2 = 0.999$ for mouse plasma. The accuracy and precision of each individual calibrator as summarized in Table 2, were $\leq 3\%$ and $\leq 6\%$ in both human and mouse plasma.

The LLOQ of the method was defined by the lowest calibrator (0.500 ng/mL) of the calibration curve, which was confirmed by measuring HMBA from six lots of human plasma samples and six lots of mouse plasma samples. The precision and accuracy of each lot of plasma at LLOQ were calculated based on five separate samples with one injection per sample. The data are summarized in Table 3. The accuracy and the precision of the method at the LLOQ

were $\leq 8\%$ and $\leq 3\%$ in human plasma, and $\leq 6\%$ and $\leq 9\%$ in mouse plasma, respectively.

Precision, accuracy, and dilution studies

Inter-assay precision and accuracy were assessed by five parallel injections from five identical QC samples at each concentration over three separate days of analysis. Intra-assay precision and accuracy were assessed by five parallel injections from five identical QC samples at each concentration. Accuracy was expressed as percent relative error (%E), and precision was determined as percent standard deviation or coefficient of variation (%CV). As shown in Table 4, the intra- and inter assay accuracy and precision were $\leq 9\%$ and $\leq 10\%$ for both human and mouse plasmas.

Since the upper limits of the linear calibrations curve were 100 ng/mL, sample concentrations beyond these concentrations were subject to dilution studies. In this work, dilution effect was assessed by 1:100 dilution of plasma QCs at the concentrations of 150, 3.00×10^3 , and 9.00×10^3 ng/mL by the pooled blank plasma, with the data summarized in Table 5. As shown in the table, the dilution study had an accuracy of $\leq 10\%$ and precision of $\leq 3\%$ over the concentration range studied. These results indicated that dilution of plasma samples which had concentrations beyond the upper limit of the calibration curve would not produce significant error in the measurement of actual HMBA concentrations.

Stability

The stability of HMBA was determined by comparing the mean-peak-area ratios of HMBA to the IS in the QC samples to those of freshly prepared QCs, expressed in terms of recovery. As shown in Table 6, the recoveries of QC samples were 98–106%, 97–107%, and 91–103% for the bench top, the autosampler, and the freeze-and-thaw studies, respectively. These studies indicated that there was no significant deviation in the quantitation of HMBA under the experimental conditions.

Table 4
Intra- and inter-accuracy and precision of HMBA in plasma.

	Intra-run				Inter-run		
	Nominal [HMBA] (ng/mL)	Measured [HMBA] ^a \pm SD (ng/mL)	Accuracy (%E)	Precision (%CV)	Measured [HMBA] ^b \pm SD (ng/mL)	Accuracy (%E)	Precision (%CV)
Human	1.50	1.59 \pm 0.03	6	2	1.46 \pm 0.14	-3	10
	30.0	31.2 \pm 0.6	4	2	29.5 \pm 1.6	-2	6
	90.0	87 \pm 3	-3	3	82 \pm 4	-9	5
Mouse	1.50	1.60 \pm 0.02	7	1	1.44 \pm 0.14	-4	10
	30.0	31.6 \pm 0.8	5	2	32.1 \pm 1.1	7	3
	90.0	94 \pm 1	4	1	94 \pm 4	4	4

^a Each datum point calculated by five parallel measurements from five identical QCs.

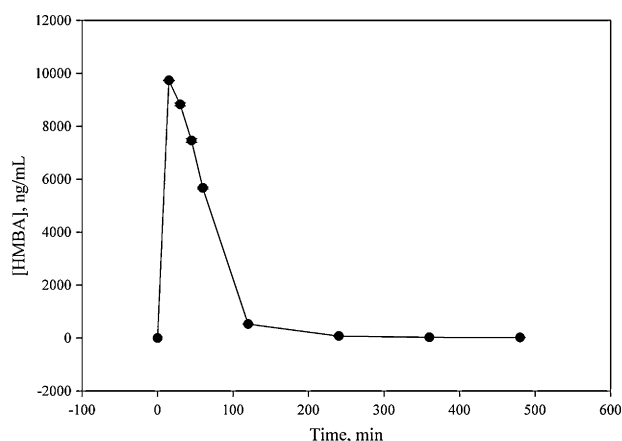
^b Each datum point calculated by five parallel measurements from five identical QCs of three different days.

Table 5
Dilution studies.

	Initial [HMBA] (ng/mL)	Dilution factor	Nominal [HMBA] (ng/mL)	Measured [HMBA] ± SD (ng/mL)	Accuracy (%E)	Precision (%CV)	Actual [HMBA] (ng/mL)
Human (n=3)	150	100	1.50	1.51 ± 0.05	1	3	151
	3.00 × 10 ³	100	30.0	29.2 ± 0.2	-3	1	2.92 × 10 ³
	9.00 × 10 ³	100	90.0	92.9 ± 1.0	3	1	9.29 × 10 ³
Mouse (n=3)	150	100	1.50	1.52 ± 0.04	1	3	152
	3.00 × 10 ³	100	30.0	31.9 ± 0.8	6	2	3.19 × 10 ³
	9.00 × 10 ³	100	90.0	98.9 ± 1.6	10	2	9.89 × 10 ³

Table 6
Stability of HMBA in plasma samples.

	Nominal [HMBA] (ng/mL)	4 h	8 h	24 h	3 Freeze-thaw cycles	
		Recovery ± SD (%)	Recovery ± SD (%)	Recovery ± SD (%)	Recovery ± SD (%)	
Bench-top stability (before deproteinization) (n=3)	Human 1.50	101 ± 5	100 ± 5	101 ± 7	91 ± 1	
		30.0	106 ± 4	104 ± 4	105 ± 2	100 ± 2
		90.0	105 ± 3	104 ± 1	104 ± 1	103 ± 1
	Mouse 1.50	102 ± 1	102 ± 2	103 ± 2	103 ± 2	
		30.0	99 ± 1	98 ± 2	99 ± 1	97 ± 0.4
		90.0	100 ± 4	102 ± 4	101 ± 3	101 ± 4
Autosampler stability (after deproteinization) (n=3)	Human 1.50	102 ± 2	100 ± 4	107 ± 4		
		30.0	102 ± 2	100 ± 3	101 ± 3	
		90.0	104 ± 0.1	103 ± 2	103 ± 0.3	
	Mouse 1.50	100 ± 3	101 ± 1	98 ± 1		
		30.0	100 ± 1	103 ± 1	97 ± 1	
		90.0	101 ± 5	103 ± 2	97 ± 3	

**Fig. 3.** Mean concentrations of HMBA in mice over time.

Application to animal study

The validated LC-MS/MS method was applied to the measurement of HMBA in FVB mice. In this work, mouse plasma samples collected by the procedure described in Section 2.6 together with eight calibrators (*i.e.*, one single-blank, one double-blank and six nonzero) and a set of QCs at low-, mid- and high-concentrations (*i.e.*, 1.50, 30.0, 90.0 ng/mL) were thawed at room temperature. These samples were prepared by the procedures described in Section 2.7, and analyzed by the validated method. The samples of concentrations beyond the upper limit of calibration curve (*i.e.*, 100 ng/mL) were re-run by 1:100 dilution using the pooled blank mouse plasma together with the dilution QC at the concentration of 9.00×10^3 ng/mL. Fig. 3 shows the HMBA concentration-time profile in FVB mice after a niple injection of 10 mg/kg. Each datum point was based on duplicate measurement of a blood sample from an FVB mouse. Although a higher dose of HMBA was used in this preliminary study, the concentration-time profile of

HMBA demonstrated not only the applicability of the method in its intended sample matrix, but also its feasibility for a wide concentration range of HMBA in plasma (from sub ng/mL to high $\mu\text{g/mL}$). This method will be used in the future delayed release dosing regimen which should have much lower plasma HMBA concentration profile.

Conclusions

This work detailed the development and validation of a LC-MS/MS method for the quantitation of HMBA in human and mouse plasma. The method used a simple deproteinization step for sample preparation, and a reversed-phase chromatographic column for analyte separation. It has a linear calibration range of 0.500–100 ng/mL and stability for routine analysis. The method was successfully applied to the measurement of HMBA in mouse plasma samples. It may be useful for the toxicokinetic study of HMBA in mice as well as pharmacokinetic study in humans.

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