Determination of 6-benzylthioinosine in Mouse and Human Plasma by Liquid Chromatography–Tandem Mass Spectrometry

Lan Li  
_Cleveland State University_

Yan Xu  
_Cleveland State University_, y.xu@csuohio.edu

David N. Wald  
_Case Western Reserve University_

William Tse  
_Case Western Reserve University_

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Introduction

6-Benzylthioinosine (6BT) is an adenosine analogue (Fig. 1) and was originally used in the property studies of adenosine amino-hydrolase [1]. In the later 1990s, 6BT was found to be potentially useful in the treatment of toxoplasmosis [2,3] caused by Toxoplasma gondii, an intracellular parasite that infects humans and many other warm-blooded animals [4,5]. Recently, 6BT was identified as a promising differentiation-inducing agent for leukemic cells as less toxic and more efficacious treatment for acute myeloid leukemia (AML) [6].

According to statistics by American Society of Clinical Oncology in 2008, AML is the most common form of acute leukemia in the United States and is most often observed in elderly population over age 65 [7,8]. The molecular pathogenesis of AML is generally accepted as a combination of differentiation arrest and the uncontrolled proliferation of the myeloblasts [9]. The standard chemotherapy agents such as Adriamycin and cytarabine work through nonselectively killing highly proliferative cells leading to significant toxicities [8]. Although up to 75% of the patients can achieve complete remission (CR), the prognosis of the patients remains poor leading to an average 5-year survival rate as low as 21% [7]. In contrast to the traditional chemotherapeutics that are used for the majority of AML patients, the treatment of acute promyelocytic leukemia (APL, a rare subtype of AML), utilizes a differentiation-inducing agent, all-trans retinoic acid (ATRA), that increases the 5-year disease free survival rate of the patients to 74% through inducing the terminal differentiation of the myeloblasts [10]. ATRA can be effectively used in combination with low dose chemotherapeutic agents without significant toxicity [11]. This differentiation approach is especially desirable for elderly patients who are often not able to tolerate the toxicities of the traditional chemotherapeutics.

Since ATRA is only useful for AML patients exhibiting the rare APL subtype, to search for efficacious differentiation-inducing agents for the treatment of other AML subtypes, Wald et al. [6] carried out a cell-based compound-library screen. 6BT has been identified as a promising differentiation-inducing agent that not only displays high differentiation-inducing activity to myeloid leukemia cell lines (i.e., HL-60 and OCI-AML3) and primary cells...
of AML patients, but also induces cell death to a subset of AML cell lines (i.e., HNT34 and MV4-11). Furthermore, 6BT exhibits very low toxicity to non-malignant cells (i.e., fibroblasts, normal bone marrow, and endothelial cells). In mouse xenograft studies, 6BT significantly decreased the tumor (HL-60) size and prevented tumor (HL-60 and MV4-11) formation in pretreated mice. Flow cytometric analysis of the dissected tumors showed a 129% increase of CD11b (a mature myeloid marker) in 6BT-treated tumors in comparison to the untreated ones [6].

The preclinical studies of 6BT for the treatment of AML and toxoplasmosis demonstrated its high potential to be an investigational new drug and warrants further therapeutic development. Therefore, a quantitative analytical method for 6BT is needed for pharmacological and toxicological studies. Nevertheless, a recent search by SciFinder® Scholar revealed that there is no analytical method available to date for quantification of 6BT in biological matrices. Only a qualitative LC–UV method for 6BT and its analogues was reported by Rais et al. [12], which was not validated for quantitative measurement of 6BT and did not have the sensitivity and specificity required for pharmacokinetic study of 6BT.

Given the above considerations, we have developed a novel LC–MS/MS method for the direct quantification of 6BT in both mouse and human plasma using 2-amino-6-benzylthioinosine (2A6BT) (Fig. 1) as the internal standard (IS). A liquid–liquid extraction procedure was developed for plasma sample preparation, and analytes were separated by YMC ODS-AQ® column using 0.1% formic acid, 45% acetonitrile and 54.9% deionized water (v/v/v) as mobile phase. The eluates from the chromatographic column were detected by tandem mass spectrometer at the positive ionization mode (ESI⁺–MS/MS). The method developed fills the gap of lacking analytical method for quantitative measurement of 6BT, which has been validated in both mouse and human plasma according to the US Food and Drug Administration (FDA) guidelines [13], and applied to a preliminary pharmacokinetic study of 6BT in mice.

Experimental

Chemicals and solutions

6BT and 2A6BT (IS) were kindly provided by the Developmental Therapeutics Program of the National Cancer Institute (Bethesda, MD, USA). Dimethylsulfoxide (DMSO), HPLC-grade of acetonitrile and ethyl acetate were purchased from Sigma–Aldrich (St. Louis, MO, USA). Formic acid was from Acros (Morris Plains, NJ, USA). Deionized water was obtained from the Barnstead NANOpure® water purification system (Thermo Scientific, Waltham, MA, USA). Pooled blank mouse plasma was purchased from Equitech-Bio (Kerrville, TX, USA). Pooled blank human plasma was from Haemtech, Inc. (Essex Junction, VT, USA). Isoflurane was purchased from Baxter Healthcare Corporation (Deerfield, IL, USA).

6BT and IS stock solutions were prepared and kept as follows: each compound was weighted out accurately by an analytical balance and dissolved into an appropriate amount of acetonitrile to a concentration of 1.00 mg/mL; then, each stock solution was pipetted separately into 1.5-mL microcentrifuge tubes at 100 μL/tube for 6BT and 25.0 μL/tube for IS. The microcentrifuge tubes containing either 6BT (100 μg/tube) or IS (25.0 μg/tube) stock solution were dried by vacuum evaporation in a DNA120 SpeedVac® (ThermoSavant, Hollbrook, NY, USA) at 25 °C for 10 min, and kept at −20 °C until use. When used, 1.00 mL of deionized water was added to each tube to make 6BT and IS working solutions at 100 and 25.0 μg/mL, respectively. The working solutions of 6BT and IS were freshly prepared daily.

The mobile phase for chromatographic separation was prepared by mixing 0.1% formic acid, 45.0% acetonitrile and 54.9% deionized water (v/v/v).

Instrumentation

The LC–MS/MS system consisted of an Agilent (Santa Clara, CA, USA) 1100 HPLC and a Micromass (Manchester, UK) Quattro II triple quadrupole mass spectrometer. The HPLC unit consisted of two binary pumps, a degasser, an autosampler, an inline filter (0.5 μm pore) (Upchurch Scientific, Oak Harbor, WA, USA), a Waters (Milford, MA, USA) YMC-AQ® column (2.0 mm × 50 mm, 5 μm particle size with 120 Å pore size), a two-position 6-port switching valve (Alltech, Deerfield, IL, USA), and a post-column splitter (Valco, Houston, TX, USA). The LC–MS/MS system operation, data acquisition and processing were done by Micromass MassLynx software (version 3.3). For each analysis, 20 μL of the sample was injected into the system. Chromatographic separation was carried out by isocratic elution using the mobile phase at the flow rate of 100 μL/min. By programming of the post-column switching valve, the first 2-min column eluate was diverted to the waste and the later eluate was detected by the triple quadrupole mass spectrometer after a post-column split (1:3).

The mass spectrometer was operated at the positive-electrospray-ionization (ESI⁺) mode. It was tuned by infusion of a mixture of 6BT (10.0 μg/mL) and IS (10.0 μg/mL) in the mobile phase at a flow rate of 3 μL/min with a syringe pump (Harvard Apparatus, South Natick, MA, USA). The optimized ionization conditions were as follows: drying gas 300 L/h, nebuliser 15 L/h, capillary voltage 3.5 kV, HV lens 0.5 kV, cone voltage 25 V, skimmer 1.5 V, RF lens 0.2 V, ion source temperature 40 °C, ion energy 0.3 V. Analyte quantification was based on multiple-reaction-monitoring (MRM) with the conditions set as follows: argon collision gas 2.0–2.5 μbar, collision energy 17 eV, dwell time 0.4 s, inter-scan delay 0.05 s, low- and high-mass resolution 15 (for both quadrupoles 1 and 3), and multiplier 650.

![6-Benzylthioinosine (6BT), MW = 374](image1)

![2-Amino-6-benzylthioinosine (2A6BT, IS), MW = 389](image2)

**Fig. 1.** The chemical structures of 6BT and the internal standard.
Preparation of standard solutions, plasma calibrators and controls, and mouse plasma samples

6BT standard solutions (6.00, 18.0, 20.0, 60.0, 180, 200, 600, 1800, 2000, and 3200 ng/mL) and IS standard solution (100 ng/mL) were prepared by serial dilution of 6BT working solution (100 μg/mL) and IS working solution (25.0 μg/mL), respectively.

6BT mouse and human plasma calibrators (3.00, 10.0, 30.0, 100, 300, and 1000 ng/mL) were prepared individually by mixing 100 μL of pooled blank plasma, 50.0 μL of 6BT standard solution (at twice of the calibrator’s concentration) and 50 μL of IS standard solution at 100 ng/mL.

6BT mouse plasma controls (3.00, 9.00, 90.0, 900, and 1600 ng/mL) and 6BT human plasma controls (3.00, 90.0 and 900 ng/mL) were prepared individually by mixing 100 μL of pooled blank plasma, 50.0 μL of 6BT standard solution (at twice of the control’s concentration) and 50 μL of IS standard solution at 100 ng/mL.

Mouse plasma samples of 6BT pharmacokinetic study (see Section 2.6) were prepared by mixing 100 μL of plasma from mice injected with 6BT, 50.0 μL of deionized water and 50.0 μL of IS standard solution at 100 ng/mL.

Liquid–liquid extraction of 6BT from plasma matrices

Plasma calibrators, controls and animal samples prepared as described in Section 2.3 were extracted using the following protocol: 1 mL of ethyl acetate was added into a 1.5 mL microcentrifuge tube containing plasma sample. After vortexing for 1 min, the mixture was centrifuged at 13,000 × g for 10 min. Then, 850 μL of the organic phase (ca. 85% of the upper layer) was transferred into a standard solution at 100 ng/mL.

6BT mouse plasma controls (3.00, 9.00, 90.0, 900, and 1600 ng/mL) and 6BT human plasma controls (3.00, 90.0 and 900 ng/mL) were prepared individually by mixing 100 μL of pooled blank plasma, 50.0 μL of 6BT standard solution (at twice of the calibration’s concentration) and 50 μL of IS standard solution at 100 ng/mL.

Results and discussion

Method development

Mass spectrometric detection

In this work, the optimization of 6BT and 2A6BT (IS) responses was done using the “auto-tune” function of the MassLynx. Since 6BT and the IS were more easily to form protonated species than deprotonated species by electrospray ionization, the positive-electrospray-ionization mode was used for 6BT identification and quantification. As shown in Fig. 2A and B (the m/z scan by the first quadrupole), 6BT and the IS produced predominant molecular ions at m/z 375 for [6BT+H]+ and m/z 390 for [IS+H]+, respectively. These molecular ions produced were further dissociated into product ions by collision with argon gas in the second quadrupole (Fig. 2C and D). The predominant product ions of [6BT+H]+ and [IS+H]+ were m/z 243 and m/z 258, respectively. Therefore, the mass transition pairs m/z 243 > 243 and m/z 258 > 258 for 6BT and the IS were chosen for quantification by multiple-reaction-monitoring (MRM) mode.

The major fragments of 6BT and the IS were proposed by comparing the chemical structures of these compounds together with the m/z differences between the predominant product ions and shown in Fig. 3.
Fig. 2. The mass spectra of 6BT and the internal standard. The experimental conditions were the same as those described in Section 2.2.

Fig. 3. The proposed major fragments of 6BT and the internal standard.
reasonable retention time, symmetrical peaks, and sufficient resolution for 6BT and 2A6BT. However, YMC ODS-AQ column was chosen for the method development because it produced higher signal response than that of Gemini column.

Because 6BT and the IS are basic compounds, addition of formic acid (0.1%) in the mobile phase could not only protonate the analytes to enhance the sensitivity of MS detection, but also increase the hydrophilicity of the analytes to reduce the retention times of the analytes on the analytical column.

In this work, the percent content of acetonitrile in the mobile phase had also been optimized in terms of separation efficiency. A complete baseline resolution of 6BT and 2A6BT was achieved with a total run time of 7.0 min using a mobile phase containing 35.0% of acetonitrile, 0.1% of formic acid and 64.9% of deionized water (v/v/v). To shorten the total run to 5.0 min, the percent content of acetonitrile in the mobile phase was increased to 45.0%, which resulted in the elution times of 2A6BT and 6BT at 3.5 and 3.9 min, respectively (Fig. 4). Although only a partial separation of between 6BT and 2A6BT was realized using 45.0% of acetonitrile in the mobile phase, these two compounds were completely separated from the sample matrix and quantified by MRM at different m/z. Therefore, a mobile phase containing 45.0% v acetonitrile, 0.1% of formic acid and 54.9% of deionized water (v/v/v) was adopted in the analytical procedure of the method, which not only improved the separation efficiency by nearly 30% but also showed no interference in 6BT quantitation.

**Method validation**

Method developed was validated by following the FDA guidelines for industry bioanalytical method validation [13].

**Matrix effect and recovery**

In this work, triplicate measurements were carried out at each concentration level for matrix effect and recovery studies. The effect (either suppression or enhancement) of sample matrix on analytical signal of 6BT was assessed by the IS normalized matrix factor (MF$_{IS}$) [14].

$$MF_{IS} = \frac{(A_{analyte}/A_{IS})_{matrix}}{(A_{analyte}/A_{IS})_{solvent}}$$

where $(A_{analyte}/A_{IS})_{matrix}$ is the peak area ratio of 6BT to the IS in SAE plasma matrix standard; and $(A_{analyte}/A_{IS})_{solvent}$ is the peak area ratio of 6BT to the IS in corresponding aqueous standard. As seen in Table 1, the MF$_{IS}$ in mouse and human plasma matrices ranged 1.10–1.13 and 0.97–1.04, respectively. The values of MF$_{IS}$ were quite reproducible with standard deviation (SD) ≤ 0.06 and consistent over the concentration range with a deviation of −0.03 to +0.13 from its ideal value of 1.00 (i.e., no matrix effect).

The IS normalized recoveries (R$_{IS}$) of 6BT from plasma samples were calculated by the following equation.

$$R_{IS} = \frac{(A_{6BT}/A_{IS})_{plasma}}{(A_{6BT}/A_{IS})_{matrix}} \times 100\%$$

where $(A_{6BT}/A_{IS})_{plasma}$ was the peak area ratio of 6BT to the IS in a plasma calibrator; and $(A_{6BT}/A_{IS})_{matrix}$ was the peak area ratio of 6BT to IS in the corresponding SAE standard. The recovery data were summarized in Table 1, which had values of 82–87% from mouse plasma and 90–98% from human plasma.

The absolute recoveries (R) of the IS from plasma samples were calculated by the following equation.

$$R = \frac{(A_{IS})_{plasma}}{(A_{IS})_{matrix}} \times 100\%$$

where $(A_{IS})_{plasma}$ and $(A_{IS})_{matrix}$ were the peak areas of the IS in a plasma calibrator and the corresponding SAE standard. The values of R ranged 59–66% and 60–64% in mouse and human plasma, respectively. Although the absolute recovery of the IS was not high, it was consistent within the calibration range.

**Calibration curve, accuracy, and precision**

6BT calibration curves in both mouse and human plasma were constructed using six non-zero plasma calibrators, one single-blank plasma (with IS only), and one double-blank plasma (with neither 6BT nor IS). The concentrations of 6BT in the non-zero samples were 3.00, 10.0, 30.0, 100, 300 and 1000 ng/mL. The linear calibration ranges (3.00–1000 ng/mL) were established in mouse and human plasma by plotting the peak area ratios of 6BT to the IS $(A_{6BT}/A_{IS})$ versus the concentrations of 6BT using 1/x (the reciprocal of 6BT concentration) weighted linear regression, and the calibration equations were given in Table 2. In this work, the lower limits
Table 2
Calibration equations of 6BT in mouse and human plasma.

<table>
<thead>
<tr>
<th>Plasma matrices</th>
<th>Actual [6BT]</th>
<th>Measured [6BT]</th>
<th>SD</th>
<th>Accuracy (%)</th>
<th>Precision (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>3.00</td>
<td>2.84</td>
<td>0.04</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>9.8</td>
<td>0.2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>30.0</td>
<td>30.3</td>
<td>0.9</td>
<td>−1</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>101</td>
<td>4</td>
<td>−1</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>301</td>
<td>9</td>
<td>−0.2</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>1.00 × 10³</td>
<td>1.00 × 10³</td>
<td>0.01 × 10³</td>
<td>0.2</td>
<td>1</td>
</tr>
<tr>
<td>Human</td>
<td>3.00</td>
<td>2.78</td>
<td>0.09</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>9.8</td>
<td>0.3</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>30.0</td>
<td>29.3</td>
<td>0.7</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>102</td>
<td>1</td>
<td>−1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>3.1 × 10²</td>
<td>0.2 × 10³</td>
<td>−4</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>1.00 × 10³</td>
<td>9.9 × 10²</td>
<td>0.2 × 10³</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

Calibration equations: Mouse plasma, \( Y = 0.020 (±0.002)X + 0.007 (±0.002) \), \( R² = 1.00 (±0.00) \), Human plasma, \( Y = 0.017 (±0.002)X + 0.003 (±0.001) \), \( R² = 0.995 (±0.002) \). Each datum point was based on three separate measurements in different days.

Table 3
Accuracy, intra- and inter-assay precisions of 6BT in mouse and human plasma.

<table>
<thead>
<tr>
<th>Plasma matrices</th>
<th>LLOQ</th>
<th>IQC</th>
<th>MQC</th>
<th>HQC</th>
<th>DQC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse Accuracy (%) n=5</td>
<td>1</td>
<td>4</td>
<td>−3</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Intra-assay precision, CV (%) n=5</td>
<td>4</td>
<td>6</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Inter-assay precision, CV (%) n=3</td>
<td>4</td>
<td>5</td>
<td>5</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Human Accuracy (%) n=5</td>
<td>−13</td>
<td>−0.6</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intra-assay precision, CV (%) n=5</td>
<td>3</td>
<td>9</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Inter-assay precision, CV (%) n=3</td>
<td>11</td>
<td>2</td>
<td>1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The concentrations of 6BT in the LLOQ, IQC, MQC, HQC and DQC of mouse plasma were 3.00, 9.00, 90.0, 900 and 1.60 × 10³ ng/mL, respectively. For the human plasma study, LQC, MQC, and HQC were 3.00, 90.0 and 900 ng/mL, respectively. The concentration of the IS was 50.0 ng/mL.

of quantification (LLOQ) were defined by lowest plasma calibrators of the calibration curves.

With the calibration equation, 6BT concentrations of the non-zero calibrators were obtained, and the accuracy as percent error was calculated. The precision as percent standard deviation or coefficient of variation (CV) was determined by replicate measurements. The results were summarized in Table 2, where accuracy ranged 0.2–5% and 1–7% and precision was 1–4% and 1–7% in mouse and human plasma, respectively. These values were well within the FDA guidelines (i.e., ≤±15% at all concentrations except at LLOQ where ≤±20%).

The intra- and inter-assay precisions were further determined using five replicates of plasma control samples at low-, mid- and high-concentration levels (LQC, MQC and HQC). Since the LQC was different from LLOQ in mouse plasma study and one sampling point in mouse pharmacokinetic study exceeded the upper limit of quantification (ULOQ), the accuracy and precision of LLOQ and dilution quality control (DQC) for mouse plasma study were also included. The intra-assay precision was by the percent peak area ratios of 6BT to the IS of the five-replicate QC samples at each concentration level and the inter-assay precision was determined as by the percent peak area ratios of 6BT to the IS by triplicate measurements of

Table 4
Stability data of 6BT under various test conditions.

<table>
<thead>
<tr>
<th>Storage conditions</th>
<th>Plasma matrices</th>
<th>[6BT] added (ng/mL)</th>
<th>Recovery ± SD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Room temperature, 4 h</td>
<td>Mouse 9.00</td>
<td>88 ± 5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Human 900</td>
<td>94 ± 2</td>
<td></td>
</tr>
<tr>
<td>Room temperature, 8 h</td>
<td>Mouse 9.00</td>
<td>102 ± 7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Human 900</td>
<td>102 ± 2</td>
<td></td>
</tr>
<tr>
<td>Room temperature, 24 h</td>
<td>Mouse 9.00</td>
<td>68 ± 7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Human 900</td>
<td>87 ± 3</td>
<td></td>
</tr>
<tr>
<td>Freeze–thaw (three cycles, −20°C)</td>
<td>Mouse 900</td>
<td>19 ± 2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Human 900</td>
<td>30 ± 2</td>
<td></td>
</tr>
<tr>
<td>Long-term (30 days, −20°C)</td>
<td>Mouse 900</td>
<td>106 ± 10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Human 900</td>
<td>100 ± 4</td>
<td></td>
</tr>
</tbody>
</table>

Each datum point was based on triplicate measurements.
Representative MRM chromatograms of mouse plasma samples. (A) pre-dosed mouse plasma with IS, (B) the plasma sample collected 5 min after intraperitoneal injection with IS, and (C) the plasma sample collected 13 min after intraperitoneal injection with IS.

A single QC sample at each concentration level, which ranged 3–6% and 0.6–11% in mouse and human plasma, respectively (Table 3). The accuracies of the above studies were −13 to 5% (Table 3), which were well within the FDA guidelines.

**Stability**

The stabilities of 6BT in mouse and human plasma were tested at two concentration levels (9.00 and 900 ng/mL) with triplicate measurements, and the results expressed as recovery were summarized in Table 4. As seen in Table 4, there was no significant loss of 6BT observed in human plasma under the tested conditions with recovery of 97–108%. For 6BT mouse plasma samples, they were stable at room temperature up to 4 h and after three freeze (−20 °C) and thaw (room temperature) cycles with recovery of 88–106%. Therefore, 6BT stock solutions and plasma samples were kept at −20 °C for this work, and the analysis of mouse plasma samples was done within a 4-h timeframe.

**Method application**

The validated LC–MS/MS method was applied to a preliminary pharmacokinetic study of 6BT in mice. Mouse plasma samples collected by the procedure described in Section 2.7 were thawed in room temperature. The mouse plasma samples together with eight calibrators (i.e., one single-blank, one double-blank and six non-zero) and two sets of QC at low-, mid- and high-concentrations (i.e., 9, 90, 900 ng/mL) were extracted after adding the IS solution and analyzed by the validated method. The sample having a concentration beyond the ULOQ (i.e., 1000 ng/mL) had been re-run along with the dilution QC (DQC) at the concentration of 1600 ng/mL after 1:1 dilution with blank mouse plasma.

Representative mass chromatograms of mouse plasma samples were shown in Fig. 5, where no sign of interference from endogenous compound in mouse plasma was observed. The 6BT concentration in mouse plasma after single bolus injection was illustrated in Fig. 6, which fitted well in a nonlinear one-compartment first-order pharmacokinetic model that has the following estimated parameters with percent relative standard deviations of 12–15%: $T_{max}$: 18.9 min; $C_{max}$: 1086 ng/mL; $T_{1/2}$: 13.1 min; and AUC: 55,731 min ng/mL.

**Conclusions**

An LC–MS/MS method for the quantitative determination of 6BT in mouse and human plasma has been developed and validated. Analytes in plasma were first extracted by ethyl acetate and then separated by Waters VMC ODS-AQ® column prior to the tandem mass spectrometric detection. Method validation has been carried out according the FDA guideline. This method had a linear calibration range of 3.00–1000 ng/mL for 6BT in both mouse and human plasma, and has been successfully applied to the pharmacokinetic study of 6BT in mice. It may be useful in therapeutic development of 6BT and its analogues in human.

**References**