Journal of Pharmaceutical and Biomedical Analysis 56 (2011) 778–784

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Development of a robust and sensitive LC–MS/MS method for the determination of adenine in plasma of different species and its application to *in vivo* studies

Jianxia Shi[a,*], Hua-Fen Liu[b], Jing Man Wong[a], Renee N. Huang[b], Elliott Jones[b], Timothy J. Carlson[a]

[a] Department of Pharmacokinetics and Drug Metabolism, Amgen Inc., 1120 Veterans Blvd., South San Francisco, CA 94080, United States
[b] AB SCIEX, 353 Hatch Dr., Foster City, CA 94404, United States

ARTICLE INFO

Article history:
Received 10 May 2011
Received in revised form 19 July 2011
Accepted 20 July 2011
Available online 27 July 2011

Keywords:
Adenine
Biomarker
LC–MS/MS
MTA
MTAP

ABSTRACT

A simple, robust, and sensitive liquid chromatography–tandem mass spectrometric (LC–MS/MS) method was developed for the measurement of endogenous adenine in mouse, rat, cynomolgus monkey, and human plasma. A "surrogate analyte" strategy was adopted by employing [^{13}C(U)]-adenine as the surrogate analyte. The plasma samples were processed by protein precipitation, and the extracted supernatant samples were subjected directly to LC–MS/MS analysis. The analysis was carried out in the negative ion detection mode using selected-reaction monitoring (SRM). The method achieved a lower limit of quantification (LLOQ) of 5.0 nM with a signal-to-noise ratio of 10. The intra- and inter-day assay coefficients of variation (CV) were ≤6.67% in rat plasma, and the mean recoveries and matrix effects across species and at various concentrations ranged from 88.8% to 104.2% and 86.0% to 110.8%, respectively. Using this methodology, the endogenous concentration of adenine in plasma of four species was found to range from 8.7 nM in human to 93.1 nM in cynomolgus monkey plasma. The assay was further applied to both an adenine pharmacokinetic study and a pivotal pharmacodynamic study evaluating the plasma concentration of adenine after a dose of 5′-deoxy-5′-methylthioadenosine (MTA).

1. Introduction

Adenine is a vital purine that is generated *in vivo* by two pathways, both *de novo* purine synthesis and also salvage pathway mediated through the degradation of 5′-deoxy-5′-methylthioadenosine (MTA). The enzyme methyl thioadenosine phosphorylase (MTAP) converts MTA, which is generated during the synthesis of polyamines, directly into adenine and 5-methylthioribose-1-phosphate (Fig. 1) [1]. Interestingly, the loss of MTAP activity or the deletion of the MTAP gene has been reported to occur in a wide variety of primary tumors, including acute lymphoblastic leukemia [2], non-small-cell lung cancer [3], and melanoma [4]. The deficiency of MTAP in tumor cells offers a potential promising approach for tumor-selective therapy, since MTAP activity is present in virtually all normal cells. The hypothesis is that tumor cells which lack MTAP activity are unable to salvage adenine from MTA and therefore will be more dependent on *de novo* synthesis of adenine. As a result, MTAP-deficient tumors would be sensitive to pharmacological agents that block *de novo* purine synthesis. Moreover, normal cells abundant in MTAP would be rescued from the toxicity of inhibitors of purine synthesis by administration of MTA that could provide a source of adenine [5].

Fundamental to elucidating the validity of this approach is an understanding of the availability of adenine to MTAP-deficient and MTAP-positive cells. As a result, it is necessary to be able to accurately assess the circulating concentrations of adenine *in vivo*. Several LC–UV and LC–MS/MS methods have been reported for measuring the levels of purines as well as pyrimidines in urine samples [6,7]. Indeed, levels of these compounds in the urine have been used as successful biomarkers for disease diagnosis [8]. To our knowledge, while methods have been reported for the analysis of hypoxanthine, xanthine and uric acid in plasma [9–11], no method has been reported for determining the concentration of adenine in plasma, nor has the physiological level of adenine in plasma from non-clinical species been previously reported.

One major challenge for the quantitative analysis of small molecule biomarkers comes from background interference due to the analyte itself being present in blank matrix used to prepare the calibration standard samples. Several methodologies have been reported to overcome such background interference. Quantitation of endogenous analytes can be carried out by using a calibration curve constructed from an alternative matrix, such as pure aqueous or organic solution [12,13] or pretreated matrix with the targeted analyte removed [14,15]. Using pure aqueous or organic solution as the matrix to prepare standard samples does not account for recovery of sample extraction and matrix effects during LC–MS/MS analysis and additional experiments are needed to validate the assay, while removal of the targeted analyte from the biological

* Corresponding author. Tel.: +1 650 244 2295; fax: +1 650 871 2934.
E-mail address: jianxias@amgen.com (J. Shi).

0731-7085/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2011.07.023

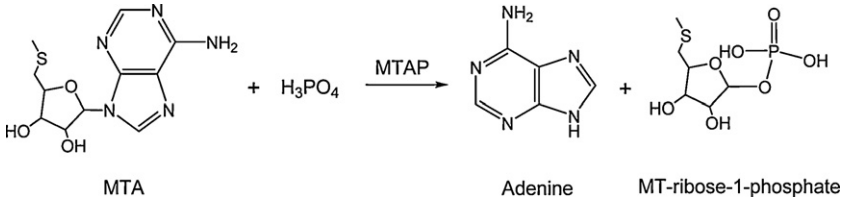

Fig. 1. Salvage pathway of purine biosynthesis through 5′-deoxy-5′-methylthioadenosine phosphate (MTAP).

matrix is generally labor intensive and is not amenable to general usage. The interference of endogenous analyte from biological matrix can also be taken out using background subtraction technique during data analysis [16]. However, the method can become impractical if the analyte level in treated subjects is lower than endogenous level. The method of standard addition [17,18] is another approach for endogenous analyte quantitation which also minimizes any matrix-related effects. This method relies on addition of a series of standard solutions to individual aliquots of the sample. The sample concentration is determined as the intercept from the calibration curve. One factor that must be considered with this method is that only samples with concentrations that are greater than the baseline matrix concentration can be accurately measured.

Generally speaking, when calibration curves are constructed in the presence of the pre-existing concentrations, the precision and accuracy of the assay at the lower range of quantitation can be compromised [12,20]. Further, the lot-to-lot variability in the pre-existing concentrations makes the method development even more difficult. In the current study, we addressed the issue of endogenous interference using an alternative approach employing a "surrogate analyte" strategy [12,18–22]. For quantitation of adenine, the stable isotope-labeled compound [^{13}C(U)]-adenine was used as the "surrogate analyte" to establish concentration–mass response relationship, i.e. a regression equation. The concentrations of the authentic analyte adenine are calculated based on the regression equations derived from the surrogate analyte [^{13}C(U)]-adenine. Because the surrogate analyte [^{13}C(U)]-adenine is not present in plasma, the assay is free from the interference caused by endogenous analytes in the matrix, which is a strong bioanalytical advantage.

Using the "surrogate analyte" approach in the current study, a simple, robust and highly sensitive LC–MS/MS method for the determination of adenine concentration in plasma was developed and validated. Compared to the previous reported method which was developed for human urine sample analysis at μM quantitation levels [6], the method presented here had an LLOQ of 5.0 nM, allowing accurate determination of the basal levels of adenine in plasma from four species. Furthermore, the method was easily applied to the determination of the pharmacokinetic profiles of adenine in mice following three different routes of administration. The method has also been used to assess concentrations of adenine in plasma after a dose of MTA, a critical pharmacodynamic result to test the rationale for targeting MTAP deficient cells [5].

2. Experimental

2.1. Materials

HPLC grade methanol, acetonitrile and water were purchased from Honeywell Burdick & Jackson (Morristown, NJ). Reagent grade ammonium formate was obtained from VWR International, LLC (West Chester, PA). Adenine, hypoxanthine, 5′-deoxy-5′-methylthioadenosine (MTA) were obtained from Sigma–Aldrich (St. Louis, MO). Stable isotope-labeled [^{13}C(U)]-adenine and [^{13}C(U)]-hypoxanthine were obtained from Moravek Biochemicals, Inc. (Brea, CA). Blank plasma samples from CD-1 mouse, Sprague–Dawley rat, cynomolgus monkey, and human were purchased from Bioreclamation, Inc. (Hicksville, NY).

2.2. Preparation of working solutions for calibration standards and quality controls

In a single 96-well plate, working solutions used for the preparation of calibration standards of adenine and [^{13}C(U)]-adenine were prepared by serial dilution of the corresponding stock solutions. Starting from 1.0 mM stock solutions in 2% methanol aqueous solution, working solutions of each analyte were prepared separately at final concentrations of 20, 50, 100, 200, 500, 1000, 2000, 5000, 10,000, and 20,000 nM using the same diluent. Corresponding working solutions for quality control samples were prepared from separate weighing powder.

2.3. Preparation of adenine and [^{13}C(U)]-adenine standard samples in water for the measurement of mass response factor

Solutions of adenine and [^{13}C(U)]-adenine at concentrations of 100, 500, 1000, 5000, and 10,000 nM were used to prepare test samples for measurement of mass response factor. Thus, 10 μL of each working solution was spiked into 100 μL water, followed by addition of 300 μL of methanol in a 96-well plate. The samples were subjected to LC–MS/MS analysis as described in Section 2.7.

2.4. Preparation of calibration standards and quality controls in plasma

Calibration standards were prepared using plasma protein precipitation method. The calibration curve consisted of 10 different concentrations of [^{13}C(U)]-adenine over the 2.0–2000 nM range. Calibration curves were prepared in plasma for each species in order to minimize the matrix effect. Using pre-prepared working solutions, 10 μL of each [^{13}C(U)]-adenine working solution was added to 100 μL of blank plasma in a 96-well plate. To precipitate plasma proteins, 300 μL of methanol containing the internal standard (100 nM [^{13}C(U)]-hypoxanthine) was added to each sample. After thorough vortex mixing for 10 min, the samples were centrifuged at 4500 rpm (~2200 × *g*, 10 °C) for 30 min. Finally, 200 μl of each plasma supernatant was then transferred to a second 96-well plate for subsequent LC–MS/MS analysis.

Quality control samples were prepared identically as the calibration standard samples. The concentrations of quality control samples were adjusted based on the calibration curves obtained from two LC–MS/MS systems (API 5000 and API 4000 Q TRAP). The working solutions of [^{13}C(U)]-adenine at concentrations of 100, 500 and 2500 nM or 500, 1000, and 5000 nM were used to yield the three QC samples with nominal concentrations at 10, 50 and 250 nM used on API 5000 system or 50, 100 and 500 nM used on API 4000 Q TRAP system.

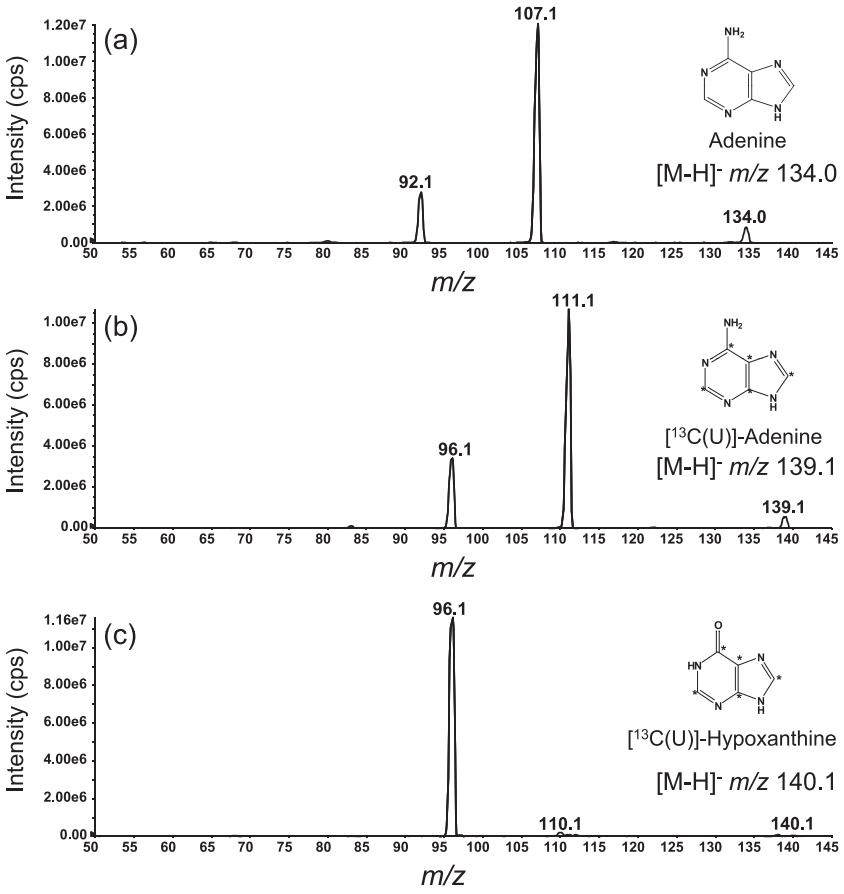

Fig. 2. Product ion spectra of adenine (top), [^{13}C(U)]-adenine (middle) and [^{13}C(U)]-hypoxanthine (bottom) following dissociation of each parent at [M−H]$^-$ obtained on API 4000 Q TRAP mass spectrometer.

2.5. Preparation of unknown plasma samples

For the quantitation of endogenous adenine, plasma samples from each species were prepared using the plasma protein precipitation method described above. To make up the volume difference between the calibration standard samples and the test plasma samples, 10 μL of 2% methanol aqueous solution was added to each 100 μL test plasma sample. The plasma samples from *in vivo* pharmacokinetic study were prepared for analysis in the same fashion.

2.6. Pharmacokinetic study of adenine

There were three treatment groups with 10 mice (NCR nude mice, Taconic Farms) per group. Animals received adenine (50 mg/kg) *via* intravenous, intraperitoneal, or subcutaneous administration. For each group, plasma samples were collected at 5, 10, 15, 30, and 60 min post-dose ($n = 2$). The samples were kept frozen at −80 °C until LC–MS/MS analysis.

2.7. LC–MS/MS conditions

Quantitative analysis of endogenous adenine levels in plasma was performed on an API 5000 triple quadrupole mass spectrometer (AB SCIEX, Foster City, CA) coupled with a Shimadzu 10ADvp pump (Shimadzu Scientific Instruments, Columbia, MD) and a CTC HTS PAL autosampler (CTC Analyticas, Switzerland). LC separation was achieved using a Shiseido Capcell PAK AQ C-18 column (5 μm, 4.6 mm × 150 mm, Shiseido Co. Ltd., Japan) with a binary gradient of 5 mM aqueous ammonium formate solution (mobile phase A) and methanol (mobile phase B). In a typical run, mobile phase was initially held at 100% mobile phase A for 1 min, followed by a linear increase of mobile phase B from 0 to 50% over 5 min. After a quick ramp of mobile phase B from 50% to 95% in 0.5 min, the solvent composition was held at 95% of mobile phase B for 2 min. The system was then returned to initial conditions for 2 min. The mobile phase flow rate was set at 800 μL/min. The sample injection volume was 10 μL for all analyses.

Negative-ion mode was used on an API 5000 mass spectrometer equipped with a turbo ion spray ion source. The instrument settings used were: source temperature (TEM) 700 °C, nebulizer gas pressure (Gas 1) 60 psi, turbo gas pressure (Gas 2) 50 psi, curtain gas pressure (CUR) 25 psi, and ion spray voltage (IS) −2000 V. Selective reaction monitor (SRM) mode was used for quantification of adenine, [^{13}C(U)]-adenine and [^{13}C(U)]-hypoxanthine (internal standard) with the following transitions, respectively: 134.0 → 107.1, 139.1 → 111.1 and 140.1 → 96.1. The dwell time for each transition was set at 100 ms with the entrance potential (EP) set at −10 V. The declustering potential (DP), collision energy (CE) and cell exit potential (CXP) were optimized at −40, −26 and −13 V for adenine, −60, −24 and −15 V for [^{13}C(U)]-adenine, and −40, −26 and −13 V for [^{13}C(U)]-hypoxanthine, respectively. The collision gas (CAD) was set at 9 arbitrary units.

Plasma samples obtained from mice following administration of adenine were analyzed using an API 4000 Q TRAP mass spectrometer (AB SCIEX, Foster City, CA) equipped with an Agilent 1200 binary pump (Agilent Technologies, Palo Alto, CA), a CTC HTS PAL autosampler. A turbo ion spray ion source was used with the following settings: TEM 600 °C, Gas 1, 50 psi, Gas 2, 50 psi, CUR 15 psi and IS at −2000 V. The DP, CE and CXP were optimized at −50, −24 and −17 V for adenine, and −35, −24 and −17 V for [^{13}C(U)]-adenine,

and −40, −22 and −15 V for [^{13}C(U)]-hypoxanthine, respectively. The collision gas was set at high.

2.8. Data analysis

Applying the surrogate analyte strategy in the quantitation, the calibration curve was generated with [^{13}C(U)]-adenine. The concentrations of adenine were determined by comparison against the calibration curve corrected with the corresponding isotopic response factor. The equation for the linear least-squares regression of the calibration curve used in the study is:

$$\text{area}_{[^{13}\text{C(U)}]-\text{adenine}}/\text{area}_{[^{13}\text{C(U)}]\text{-hypoxanthine}} = a[\text{conc}_{[^{13}\text{C(U)}]\text{-adenine}}] + b$$

where a is the slope of the regression line and b is the intercept. The concentrations of adenine in the unknown samples were calculated by

$$\text{conc}_{\text{adenine}} = \frac{[\text{area}_{\text{adenine}}/\text{area}_{[^{13}\text{C(U)}]\text{-hypoxanthine}}]\text{RF} - b}{a}$$

where RF is mass spectroscopic response factor determined by the peak areas of adenine and [^{13}C(U)]-adenine in neat solution at equivalent concentrations:

$$\text{RF} = \text{area}_{[^{13}\text{C(U)}]\text{-adenine}}/\text{area}_{\text{adenine}}$$

MultiQuant™ Software 1.0 (AB SCIEX, Foster City, CA) was used for data analysis as it allows calculation of concentrations of one analyte using the calibration curve constructed from a different analyte. Watson LIMS software (Version 7.0.0.01, Thermo Scientific) was also used employing the "Alternate Regression" function, which yielded similar results as the data calculated using MultiQuant™ Software 1.0.

3. Results and discussion

3.1. Method development

3.1.1. Surrogate analyte strategy

In consideration of availability, purity, and MS/MS fragmentation patterns, [^{13}C(U)]-adenine was chosen as the surrogate analyte for quantitative analysis of the endogenous levels of adenine in plasma. Compared to unlabelled adenine, [^{13}C(U)]-adenine generated the expected different product ions (Fig. 2) while exhibiting the same retention time under these conditions (Fig. 3). Purity of [^{13}C(U)]-adenine was greater than 99% based on the vendor's specification, and no detectable signal (greater than 5.0 nM) was observed at the retention time from adenine SRM channel using a 2.0 μM [^{13}C(U)]-adenine sample in neat solution. The level of chemical noise between the SRM channels of [^{13}C(U)]-adenine and adenine at the retention time of interest was very similar. In order to eliminate any isotope effects that could lead to different ionization efficiency, 75% methanol aqueous solutions containing an equal molar mixture of adenine and [^{13}C(U)]-adenine at concentrations of 10, 50, 100, 500 and 1000 nM were used to determine the mass spectrometer response factor (RF). The RF values were calculated by comparing the response of adenine to the response of [^{13}C(U)]-adenine at each concentration level; the values were 0.96, 0.92, 0.98, 0.95 and 1.02, respectively. The data suggested that the mass spectrometer response factor for adenine and [^{13}C(U)]-adenine was concentration-independent in the tested range. The measured RF values were very close to the theoretical value of one and this indicated that there is no difference in the ionization and fragmentation efficiency between unlabelled and stable isotope-labeled adenine. Consequently, a nominal value of 1.0 was used for quantitative analysis of adenine.

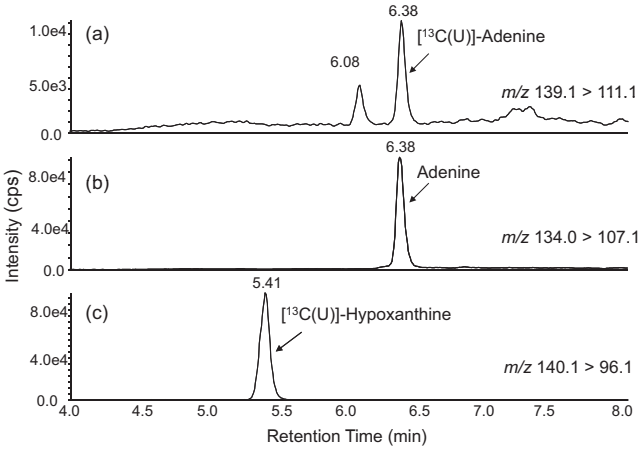

Fig. 3. SRM chromatograms of adenine, [^{13}C(U)]-adenine and [^{13}C(U)]-hypoxanthine from rat plasma containing 5 nM [^{13}C(U)]-adenine and 100 nM [^{13}C(U)]-hypoxanthine obtained on API 5000 mass spectrometer.

3.1.2. LC–MS/MS method optimization

Due to the small size and highly polar character, adenine and hypoxanthine are not easily amenable to successful chromatographic retention using regular reverse-phase C-18 or C-8 columns. This is particularly true when the analytes are in a solution containing a high percent of organic solvent (methanol or acetonitrile) which results from protein precipitation workup. Upon column screening, it was found that a Shiseido Capcell PAK AQ C-18 column (4.6 mm × 150 mm, 5 μm) with high surface polarity was able to retain both adenine and hypoxanthine injected in a methanol/water 3/1 solution. This allowed direct injection of the supernatant resulting after protein precipitation workup. Methanol was chosen as mobile phase B since it gave a slightly higher mass spectrometric signal response (∼10%) for adenine than acetonitrile, although acetonitrile provided slightly higher sensitivity (∼10%) for hypoxanthine.

The mass spectrometer was operated in negative ion mode. For each compound, $[M-H]^-$ was the predominant ion in the Q1 spectrum, which was used as the precursor ion for obtaining MS/MS product-ion spectrum. MS/MS product-ion spectra of adenine, [^{13}C(U)]-adenine and [^{13}C(U)]-hypoxanthine are shown in Fig. 3. The CAD fragmentation patterns of adenine and [^{13}C(U)]-adenine were identical to each other. For adenine, the most abundant product ion m/z 107.1 is due to loss of HCN from the precursor ion (m/z 134.0 → 107.1); this transition was considered a specific fragmentation pathway and was chosen to determine the concentration of adenine. For [^{13}C(U)]-hypoxanthine, the most abundant product ion was m/z 96.1, so the transition m/z 140.1 → 96.1 was chosen to determine the concentration of [^{13}C(U)]-hypoxanthine. Initial experiments of quantifying endogenous adenine levels in plasma indicated a five-fold increase of sensitivity with an API 5000 compared to an API 4000 Q TRAP using the optimized settings of each system. Therefore, endogenous adenine levels in plasma of four species were assayed on an API 5000 mass spectrometer.

3.2. Method validations

3.2.1. Selectivity and specificity

The selectivity and specificity of the method were ascertained through analysis of samples prepared in a methanol/water 3/1 solution or extracted from three different lots of plasma from each species. The degree of interference was assessed by inspection of

Table 1
Accuracy and precision data for [^{13}C(U)]-adenine in rat plasma.

Nominal conc. (nM)	Average (nM)	Relative error (%)	Coefficient of variation (%)
Day 1 (n = 8)			
20	21.3	6.25	3.95
50	48.7	−2.63	5.45
100	101	0.50	3.35
500	504	0.80	6.67
Day 2 (n = 8)			
20	20.3	1.31	4.32
50	47.5	−5.05	3.63
100	94.1	−5.92	5.74
500	464	−7.25	2.80
Day 3 (n = 8)			
20	19.9	−0.63	5.24
50	48.6	−2.90	3.53
100	97.0	−3.05	4.38
500	485	−3.03	4.04
Inter-day (n = 24)			
20	20.6	2.31	4.50
50	48.3	−3.53	4.20
100	97.4	−3.21	4.49
500	486	−3.16	4.50

LC–MS/MS SRM chromatograms. No significant interference peaks from the blank plasma samples of four species were found in the SRM channel of [^{13}C(U)]-adenine (t_R 6.38 min) and [^{13}C(U)]-hypoxanthine (t_R 5.41 min). The plasma extract samples from all four species contained an interference peak (t_R 6.08 min) at level of 2000 counts per second, which did not affect the quantitation of the analyte of interest.

3.2.2. The lower limit of quantification

The lower limit of quantitation (LLOQ) for [^{13}C(U)]-adenine in four species plasma was determined to be 5.0 nM (signal-to-noise ratio of 10) on an API 5000 system (Fig. 3), which was significantly lower than that reported in urine assays [5,6]. The LLOQ for [^{13}C(U)]-adenine in four species plasma was determined to be 20.0 nM (signal-to-noise ratio of 10) on an API 4000 Q TRAP system.

3.2.3. Linearity, precision and accuracy

Calibration curves of [^{13}C(U)]-adenine were obtained by analyzing eight standards in corresponding plasma matrix with the concentration range of 5–1000 nM on an API 5000 system or 20–2000 nM on an API 4000 Q TRAP system. Correlation coefficients (r^2) of calibration curves exceeded 0.990 in all batch runs. The precision and accuracy of the assay was determined by analyzing QC samples along with a calibration curve on three different days (Table 1). Intra-day performance was assessed by replicate analysis (n = 8) of these samples from two independent batch runs. Inter-day performance was evaluated by replicate analysis (n = 24) over three days of analysis. The coefficient of variation and relative error were calculated as previously described [23]. Relative errors of intra-day results ranged from −7.25% to 6.25% at the four tested concentrations, whereas coefficient of variation was between 2.80% and 6.67% (Table 1). Inter-day performance, assessed *via* replicate analysis (n = 24 per concentration) over a three day period, showed that the relative error over the 20–500 nM ranged from −3.53% to 2.31%, and the coefficient of variation was less than 4.50%.

3.2.4. Percent recovery

Extraction efficiency of the assay was assessed by comparing the response ratios of extracted QCs of [^{13}C(U)]-adenine in plasma with that of QCs prepared by spiking [^{13}C(U)]-adenine in extracted plasma using three replicates at each of the four concentration levels in plasma from each species. The recovery of [^{13}C(U)]-adenine ranged from 88.8% to 104% across the species at all the concentrations tested (Table 2). The recovery of the internal standard [^{13}C(U)]-hypoxanthine at the concentration used in the assay was assessed in a similar fashion, and the average recovery in mouse, rat, cynomolgus monkey and human plasma was 103 ± 3.3%, 94.4 ± 0.5%, 102 ± 4.2% and 101 ± 3.0%, respectively.

3.2.5. Matrix effect

The matrix effect of [^{13}C(U)]-adenine was assessed by comparing the LC–MS/MS responses of QCs prepared by spiking [^{13}C(U)]-adenine in extracted plasma with that of QCs prepared by spiking [^{13}C(U)]-adenine in a methanol/water 3/1 solution using three replicates at each of the four concentration levels in plasma from each species. The matrix effects of [^{13}C(U)]-adenine ranged from 86.0% to 111% across the species at all the concentration levels tested (Table 3). The data indicated that no coeluting endogenous substances led to any significant ion suppression, or enhancement, under the conditions of the LC–MS/MS method described. The matrix effect of the internal standard [^{13}C(U)]-hypoxanthine at the concentration used in the assay was assessed similarly, and the average matrix effect in mouse, rat, cynomolgus monkey and human plasma was 97.6 ± 1.2%, 87.4 ± 2.5%, 91.9 ± 2.5% and 98.6 ± 3.4%, respectively.

The matrix effect on adenine was evaluated due to concerns of the varied susceptibility of adenine and [^{13}C(U)]-adenine to endogenous interference. In order to minimize endogenous adenine interference, the matrix effect of adenine was assessed by comparing the responses of 1 μM adenine in 75% methanol aqueous solution with 1 μM adenine in plasma extracts from four species (n = 3). The data (not shown) indicated that the matrix effect on adenine is similar to that of [^{13}C(U)]-adenine, which provided confidence for the use of [^{13}C(U)]-adenine towards the construction of calibration curves for the quantitation of adenine.

3.3. Endogenous concentrations of adenine determined in mouse, rat, cynomolgus monkey and human plasma

Two different lots of commercially sourced plasma from each species (Bioreclamation Inc.) were assayed for adenine concentrations. The results from the analysis showed that (1) variability between different lots and (2) the endogenous levels of adenine were species-dependent. Adenine concentrations in mouse, rat and human plasma were similar (approximately 10 nM), while its concentration in cynomolgus monkey was significantly higher (above 50 nM) (Table 4). An LLOQ of 5.0 nM was achieved using an API 5000 LC–MS/MS system which provided increased confidence in the quantitation of endogenous adenine, especially for the species exhibiting relatively low levels.

3.4. Pharmacokinetic study of adenine

The present LC–MS/MS method was used to analyze plasma samples from mice that were treated with adenine. The purpose of the study was to assess the pharmacokinetic profiles of adenine following intravenous, intraperitoneal, or subcutaneous administration. The mean plasma concentration–time course profiles of adenine after a single dose of adenine at 50 mg/kg were shown in Fig. 4. The maximum plasma concentrations (C_{max}) from intravenous, intraperitoneal, and subcutaneous routes of administration were 257 ± 3.2 μM, 177 ± 8.6 μM, and 196 ± 6.9 μM, respectively. The times corresponding to maximum plasma concentrations (T_{max}) *via* the three different routes were 5-min, 5-min, and 15-min, respectively, and the areas under the plasma concentration–time curve (AUC_{0-t}) were 5310, 5170, and 5520 μM min, respectively. The results indicated that adenine was eliminated rapidly ($t_{1/2}$, 10.5 min *via* intravenous administration), and the total exposures of adenine in plasma *via* the three different routes are similar. Due

Table 2
Recovery of [^{13}C(U)]-adenine in mouse, rat, cynomolgus monkey and human plasma.

Concentration (nM)	Mouse mean ± S.D. (%)	Rat mean ± S.D. (%)	Cynomolgus monkey mean ± S.D. (%)	Human mean ± S.D. (%)
20	92.2 ± 3.6	89.4 ± 4.3	94.7 ± 7.3	104 ± 2.6
50	92.1 ± 4.4	88.8 ± 6.8	101 ± 3.5	97.4 ± 5.9
100	94.7 ± 4.8	91.3 ± 3.4	101 ± 4.0	101 ± 2.5
500	96.3 ± 0.5	93.4 ± 1.9	98.4 ± 0.6	96.3 ± 1.9

Table 3
Matrix effect of [^{13}C(U)]-adenine in mouse, rat, cynomolgus monkey and human plasma.

Concentration (nM)	Mouse mean ± S.D. (%)	Rat mean ± S.D. (%)	Cynomolgus monkey mean ± S.D. (%)	Human mean ± S.D. (%)
20	91.2 ± 2.7	93.8 ± 4.5	95.5 ± 4.2	102 ± 2.2
50	86.0 ± 1.3	88.2 ± 0.5	96.3 ± 4.3	96.8 ± 5.1
100	98.2 ± 5.1	98.4 ± 3.3	98.4 ± 2.3	110 ± 1.3
500	102 ± 2.0	102 ± 1.6	101 ± 1.9	111 ± 4.5

Table 4
Endogenous adenine levels determined in mouse, rat, cynomolgus monkey and human plasma.

	Mouse mean ± S.D. (nM)	Rat mean ± S.D. (nM)	Cynomolgus monkey mean ± S.D. (nM)	Human mean ± S.D. (nM)
Lot 1	10.1 ± 0.6	15.7 ± 0.6	93.1 ± 1.3	8.68 ± 0.6
Lot 2	5.32 ± 0.5	9.60 ± 0.7	42.8 ± 2.2	8.38 ± 0.5

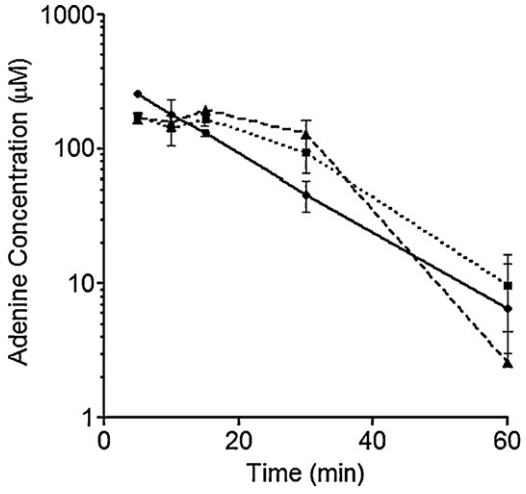

Fig. 4. Mean plasma concentration–time profiles of adenine following intravenous (—), intraperitoneal (- -), or subcutaneous (—) administration of adenine (50 mg/kg) to NCR nude mice.

to the high dosage of adenine, the levels of adenine in plasma were high compared to endogenous levels. Therefore, the *in vivo* plasma samples were diluted with water (100-fold), and the corresponding calibration standards and quality control samples were prepared in 100-fold diluted matrix also.

4. Conclusion

A simple, robust and sensitive LC–MS/MS method for the determination of endogenous adenine concentration in plasma has been established and validated. Direct injection of the supernatant after protein precipitation sample workup was followed by LC–MS/MS analysis in the negative ion mode by SRM. The surrogate analyte strategy overcame the analytical challenges of measuring endogenous levels of adenine. The method was able to determine the endogenous levels of adenine in plasma from four species, as well as adenine pharmacokinetic profiles in mice. Furthermore, the method has been applied successfully to the determination of adenine levels following treatment with MTA, and these data provided vital information regarding the evaluation of the MTAP-mediated pathway of purine biosynthesis as a pharmacological target [5].

Acknowledgements

The authors would like to acknowledge Dr. Astrid Ruefli-Brasse (Oncology Research, Amgen Inc., South San Francisco) for the useful discussions regarding MTAP, and Dr. Minqing Rong and Jessica Orf (Oncology Research, Amgen Inc., South San Francisco) for assistance in performing *in vivo* studies. The authors would also like to acknowledge Dr. Bradley Wong and Dr. Mark Grillo (Pharmacokinetics and Drug Metabolism, Amgen Inc., South San Francisco) for reviewing the manuscript.

References

[1] A.E. Pegg, H.G. Williams-Ashman, Phosphate-stimulated breakdown of 5′-methylthioadenosine by rat ventral prostate, Biochem. J. 115 (1969) 241–247.
[2] A. Batova, M.B. Diccianni, T. Nobori, Frequent deletion in the methylthioadenosine phosphorylase gene in T-cell acute lymphoblastic leukemia: strategies for enzyme-targeted therapy, Blood 88 (1996) 3083–3090.
[3] O.I. Olopade, D.L. Buchhagen, K. Malik, Homozygous loss of the interferon genes defines the critical region on 9p that is deleted in lung cancers, Cancer Res. 53 (1993) 2410–2415.
[4] J.H. Fitchen, M.K. Riscoe, B.W. Dana, H.J. Lawrence, A.J. Ferro, Methylthioadenosine phosphorylase deficiency in human leukemias and solid tumors, Cancer Res. 46 (1986) 5409–5412.
[5] A. Ruefli-Brasse, D. Sakamoto, J. Orf, M. Rong, J. Shi, T. Carlson, K. Quon, A. Kamb, D. Wickramasinghe, Methylthioadenosine rescues methylthioadenosine phosphorylase-deficient tumors from purine synthesis inhibition in vivo via non-autonomous adenine supply, J. Cancer Therapy. (2011), in press.
[6] G.L. Marca, B. Casetta, S. Malvagia, E. Pasquini, M. Innocenti, M.A. Donati, E. Zammarchi, Implementing tandem mass spectrometry as a routine tool for characterizing the complete purine and pyrimidine metabolic profile in urine samples, J. Mass Spectrom. 41 (2006) 1442–1452.
[7] S. Hartmann, J.G. Okun, C. Schmidt, C. Langhans, S.F. Garbade, P. Burgard, D. Haas, J.O. Sass, W.L. Nyhan, G.F. Hoffmann, Comprehensive detection of disorders of purine and pyrimidine metabolism by HPLC with electrospray ionization tandem mass spectrometry, Clin. Chem. 52 (2006) 1127–1137.
[8] M. Czauderna, J. Kowalczyk, Quantification of allantoin, uric acid, xanthine and hypoxanthine in ovine urine by high-performance liquid chromatography and photodiode array detection, J. Chromatogr. B 744 (2000) 129–138.
[9] M. Czauderna, J. Kowalczyk, Simultaneous measurement of allantoin, uric acid, xanthine and hypoxanthine in blood by high-performance liquid chromatography, J. Chromatogr. B 704 (1997) 89–98.

[10] D. Farthing, D. Sica, T. Gehr, B. Wilson, I. Fakhry, T. Larus, C. Farthing, H.T. Karnes, An HPLC method for determination of inosine and hypoxanthine in human plasma from healthy volunteers and patients presenting with potential acute cardiac ischemia, J. Chromatogr. B 854 (2007) 158–164.
[11] N. Cooper, R. Khosravan, C. Erdmann, J. Fiene, J.W. Lee, Quantification of uric acid, xanthine and hypoxanthine in human serum by HPLC for pharmacodynamic studies, J. Chromatogr. B 837 (2006) 1–10.
[12] M. Jemal, A. Schuster, D.B. Whigan, Liquid chromatography/tandem mass spectrometry methods for quantitation of mevalonic acid in human plasma and urine: method validation, demonstration of using a surrogate analyte, and demonstration of unacceptable matrix effect in spite of use of a stable isotope analog internal standard, Rapid Commun. Mass Spectrom. 17 (2003) 1723–1734.
[13] E. Kindt, Y. Shum, L. Badura, P.J. Snyder, A. Brant, S. Fountain, G. Szekely-Klepser, Development and validation of an LC/MS/MS procedure for the quantification of endogenous *myo*-inositol concentrations in rat brain tissue homogenates, Anal. Chem. 76 (2004) 4901–4908.
[14] J. Palandra, J. Prusakiewicz, J.S. Ozer, Y. Zhang, T.G. Heath, Endogenous ethanolamide analysis in human plasma using HPLC tandem MS with electrospray ionization, J. Chromatogr. B 877 (2009) 2052–2060.
[15] A.M. Bishop, C. Fernandez, R.D. Whitehead Jr., P. Morales-A, D.B. Barr, L.C. Wilder, S.E. Baker, Quantification of riboflavin in human urine using high performance liquid chromatography–tandem mass spectrometry, J. Chromatogr. B 879 (2011) 1823–1826.
[16] M.S. Rashed, A.A.A. Saadallah, Z. Rahbeeni, W. Eyaid, M.Z. Seidahmed, S. Al-Shahwan, M.A.M. Salih, M.E. Osman, M. Al-Amoudi, L. Al-Ahaidib, M. Jacob, Determination of urinary *S*-sulphocysteine, xanthine and hypoxanthine by liquid chromatography–electrospray tandem mass spectrometry, Biomed. Chromatogr. 19 (2005) 223–230.
[17] M. Bader, A systematic approach to standard addition methods in instrumental analysis, J. Chem. Educ. 57 (1980) 703–706.
[18] R. Ahmadkhaniha, A. Shafiee, N. Rastkari, M.R. Khoshayand, F. Kobarfard, Quantification of endogenous steroids in human urine by gas chromatography mass spectrometry using a surrogate analyte approach, J. Chromatogr. B 878 (2010) 845–852.
[19] W. Li, L. Cohen, Quantitation of endogenous analytes in biofluid without a true blank matrix, Anal. Chem. 75 (2003) 5854–5859.
[20] W. Jian, R. Edom, N. Weng, P. Zannikos, Z. Zhang, H. Wang, Validation and application of an LC–MS/MS method for quantitation of three fatty acid ethanolamides as biomarkers for fatty acid hydrolase inhibition in human plasma, J. Chromatogr. B 878 (2010) 1687–1699.
[21] H.R. Liang, T. Takagaki, R.L. Foltz, P. Bennett, Quantitative determination of endogenous sorbitol and fructose in human erythrocytes by atmospheric-pressure chemical ionization LC tandem mass spectrometry, J. Chromatogr. B 824 (2005) 36–44.
[22] N. Penner, R. Ramanathan, J. Zgoda-Pols, S. Chowdhury, Quantitative determination of hippuric and benzoic acids in urine by LC–MS/MS using surrogate standards, J. Pharm. Biomed. Anal. 52 (2010) 534–543.
[23] B.K. Wong, P.J. Bruhin, J.H. Lin, Column-switching high-pressure liquid chromatographic method for the determination of a new carbapenem antibiotic, L-739,428, in rat and monkey plasma, J. Chromatogr. B 655 (1994) 158–162.