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Development and validation of a liquid chromatography–tandem mass spectrometric method for the determination of α-methyltyrosine in human plasma

David Humphries[a,*], Kenneth Ruterbories[a], Clark Chan[b], Rangaraj Narayanan[a]

[a] *Department of Drug Disposition, Eli Lilly and Company, Lilly Corporate Center, Indianapolis, IN 46285, USA*
[b] *Lilly-NUS Centre for Clinical Pharmacology Pte Ltd., Level 6, Clinical Research Centre MD 11, National University of Singapore, 10 Medical Drive, Singapore 117597, Singapore*

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Abstract

A sensitive and selective LC–MS–MS method for the isolation and quantification of α-methyltyrosine (AMT) from human plasma is described. The method employs a simple protein precipitation using zinc sulfate and sodium hydroxide. This precipitation procedure produced samples with high aqueous content that could be directly injected into a LC–MS–MS system without compromising reverse-phase chromatographic performance. Chromatographic separation was performed on a MetaChem MonoChrom C_{18} column (2.0 mm × 50 mm; 5 μm) at a flow rate of 1 mL/min. Compounds were eluted using a gradient mixture of water–acetic acid (100:0.1, v/v) and acetonitrile–acetic acid (100:0.1, v/v). The structural analog α-hydroxymethyltyrosine was used as the internal standard. Mass spectrometric detection was carried out with a triple quadrupole mass spectrometer. The method was validated and used to determine human plasma AMT concentrations, and has been implemented to derive pharmacokinetic parameters.

Keywords: α-Methyltyrosine; Validation

1. Introduction

α-Methyltyrosine (AMT) (Fig. 1) is a white crystalline compound marketed under trade name DEMSER®. It is commonly referred to as α-methyl-*p*-tyrosine or AMPT. AMT is primarily indicated in treatment of patients with pheochromocytoma, but has also been used in the treatment of other disorders such as migraine, open angle glaucoma, and psychiatric symptoms [1–3]. It is an inhibitor of tyrosine hydroxylase, the enzyme involved in the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA). AMT inhibits catecholamine biosynthesis at the first step [3]. Hence, AMT is directly involved in inhibition of dopamine synthesis.

Prolactin is a hormone primarily synthesized in the pituitary gland. In mammals, two major sites of prolactin involvement include the mammary gland and ovary. Major functions in humans include lactation and regulation of sex steroids and reproductive function [4]. Prolactin release inhibitory factors (PIF) govern secretion of prolactin and a major player is dopamine [4]. Thus, dopamine-releasing agents such as amphetamine decrease plasma prolactin levels in rats [5]. Conversely, it is well documented that dopamine antagonists (e.g. haloperidol) cause hyperprolactinaemia due to stimulation of D2 receptors. Similarly, catecholamine synthesis inhibitors such as AMT also increase prolactin levels [5].

Previous studies have demonstrated that administration of AMT increases endogenous prolactin levels both in animals and humans [6–9]. Thus, measuring AMT concentrations in plasma could help establish AMT pharmacokinetics and also aid in potentially developing a pharmacoki-

* Corresponding author. Tel.: +1 317 277 0046; fax: +1 317 651 1332.
E-mail address: humphries_david_j@lilly.com (D. Humphries).

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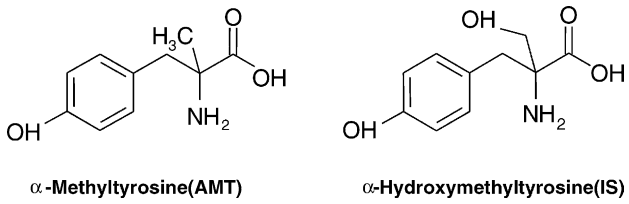

Fig. 1. Structures of AMT and IS.

netic/pharmacodynamic (PK/PD) model between AMT and prolactin. Previous approaches for measuring AMT in plasma using liquid chromatography coupled with fluorescence detection have been described in the literature [10–13]. These methods did not meet our needs for sensitivity, and due to laborious sample preparation, were not practical for analyzing large numbers of samples. We have developed a LC–MS–MS method that is robust, sensitive (lower limit of quantification (LLOQ) = 50 ng/mL) and due to short run times (1.8 min) and simple sample preparation, is amenable to high sample throughput. A large dynamic assay concentration range (50–102,400 ng/mL) was achieved with acceptable sample-to-sample carry-over. The method has been applied successfully for measuring clinically relevant AMT plasma levels.

2. Experimental

2.1. Reagents, chemicals, and blood plasma

AMT and α-hydroxymethyltyrosine (IS) (Fig. 1) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Methanol (high purity) and acetonitrile (high purity) were obtained from EM Science (Gibbstown, NJ, USA). Isopropyl alcohol (high purity) and acetic acid (glacial) were acquired from Mallinkrodt (Paris, KY, USA). Sodium hydroxide was purchased from Red Bird Service (Osgood, IN, USA). Zinc sulfate (ACS grade) was procured from Aldrich (St. Louis, MO, USA). HPLC quality water (approximately 18 MΩ) was prepared using a Millipore Milli-Q water purification system (Millipore, Milford, USA). Heparinized human plasma was obtained from Biological Specialty Corporation (Colmar, PA, USA).

2.2. Sample preparation

2.2.1. Solution preparation

Primary stock solutions were prepared by diluting accurately weighed quantities of AMT and IS to a nominal concentration of 512 μg/mL in MeOH–water–formic acid (50:50:0.1, v/v/v). Working stock solutions of AMT were prepared over the concentration range of 50–102,400 ng/mL by serial dilution of the primary stock in water–acetic acid (100:0.1, v/v). A working solution of IS (5120 ng/mL) was prepared by diluting the primary IS stock in 0.5 M NaOH. All primary stock solutions were stored at room temperature, and were stable for at least 2 weeks when stored under these conditions (Table 1).

Table 1
Stability of AMT in neat solutions and human plasma

Experiment	Duration	Difference from freshly prepared or theoretical (%)
Plasma room temperature stability (250 ng/mL; 89,600 ng/mL)	24 h	1.90
Plasma long-term stability at −20 °C (250 ng/mL; 89,600 ng/mL)	118 days	1.19
Stock solution stability (512 μg/mL)	14 days	−1.02

2.2.2. Plasma pool preparation

Validation, quality control (QC), and stability samples containing AMT were prepared as pools in human plasma at concentrations of 50, 250, 51,200, 89,600, and 102,400 ng/mL. The pooled samples were aliquoted into individual cryovials and stored at −20 °C. Samples were removed from the freezer as needed and analyzed along with freshly prepared standards for validation (50, 51,200, and 102,400 ng/mL), stability analysis (250 and 89,600 ng/mL), or sample analysis (QC samples at concentrations of 250, 51,200, and 89,600 ng/mL).

2.2.3. Extraction procedure

Calibration samples were prepared by adding 25 μL of human plasma to the wells of a 500 μL conical 96-well plate, then spiking the plasma with 25 μL of the AMT standard working solutions. The final concentrations of the AMT plasma standards were 50 (LLOQ), 100, 400, 800, 3200, 6400, 12,800, 25,600, 51,200, 76,800, and 102,400 ng/mL. Validation, QC, stability, and study samples were diluted with an equal volume of water–acetic acid (100:0.1, v/v). A 50 μL aliquot of 20% zinc sulfate (w/v) was added to all wells, followed by a 50 μL aliquot of the internal standard working solution (prepared in 0.5 M NaOH). The final IS plasma concentration was 10,240 ng/mL in all standard, validation, QC, stability, and study samples throughout the study. The samples were vortex-mixed, followed by centrifugation at 3500 rpm for 10 min. A 20 μL aliquot of the resulting supernatant was decanted and mixed with 380 μL of water–acetic acid (100:0.1, v/v) in a clean 96-well plate prior to analysis. From this resulting mixture, 20 μL was injected into the LC–MS–MS system.

2.3. Chromatographic conditions

The HPLC system consisted of a Shimadzu solvent delivery system (Kyoto, Japan), a Gilson 215 autosampler (Middleton, WI, USA), and a Rheodyne LabPro six-port valve (Rohnert Park, CA, USA) used for directing LC flow. HPLC separation was accomplished with a MetaChem MonoChrom C_{18} column (2.0 mm × 50 mm; 5 μm) purchased from Varian, Inc. (Lake Forrest, CA, USA). The HPLC separation was

performed at a flow rate of 1 mL/min (column maintained at 10 °C). The mobile phase was composed of water–acetic acid (100:0.1, v/v) (A) and acetonitrile–acetic acid (100:0.1, v/v) (B). The pH of mobile phase A was 3.2, and the observed pH of mobile phase B was 5.8. The gradient profile changed from 100% mobile phase A to 100% mobile phase B over the first 0.9 min of the sample injection, and returned to 100% mobile phase A from 0.9 to 1.2 min. Flow was diverted away from the mass spectrometer to waste for the initial 0.6 min of the sample injection.

2.4. Mass spectrometric conditions

Mass spectrometric detection was carried out using a Applied Biosystems/MDS Sciex API 3000 triple quadrupole mass spectrometer (Foster City, CA, USA) equipped with a TurboIonSpray™ interface. A Dell Dimension XPS R400 personal workstation (Round Rock, TX, USA) equipped with Applied Biosystems/MDS Sciex Analyst software (version 1.2) was used to collect and process the data.

Selected reaction monitoring $(M + H)^+$ transitions *m/z* 196 → 150 and *m/z* 212 → 166 were monitored for AMT and IS, respectively (Figs. 2 and 3). The TurboIonSpray™ temperature was maintained at 500 °C, with nebulizing gas (nitrogen) and auxiliary gas (nitrogen) flow rates at 12 and 7 L/min, respectively. The curtain gas (nitrogen) flow rate

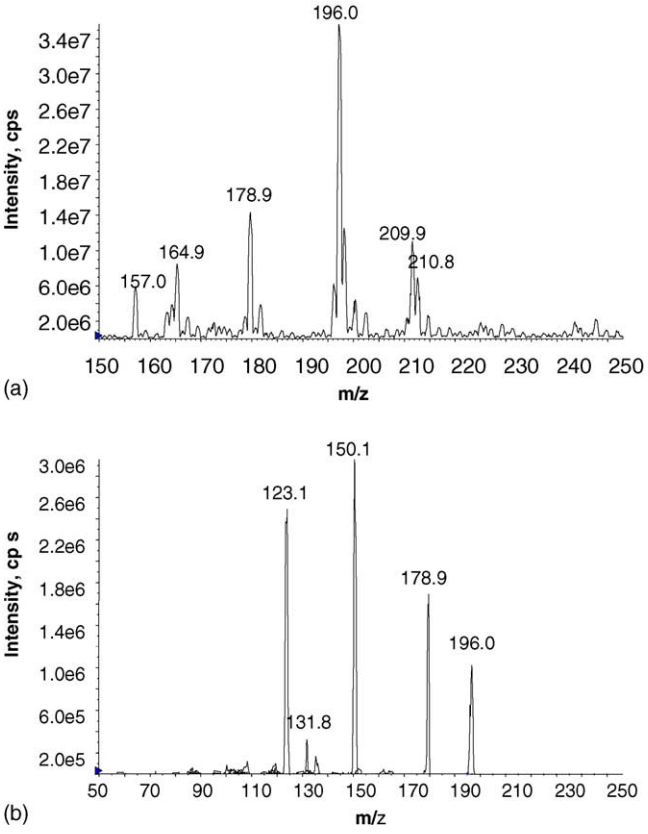

Fig. 2. AMT (*m/z* 196) positive ion electrospray mass spectrum (a) and product ion mass spectrum (b).

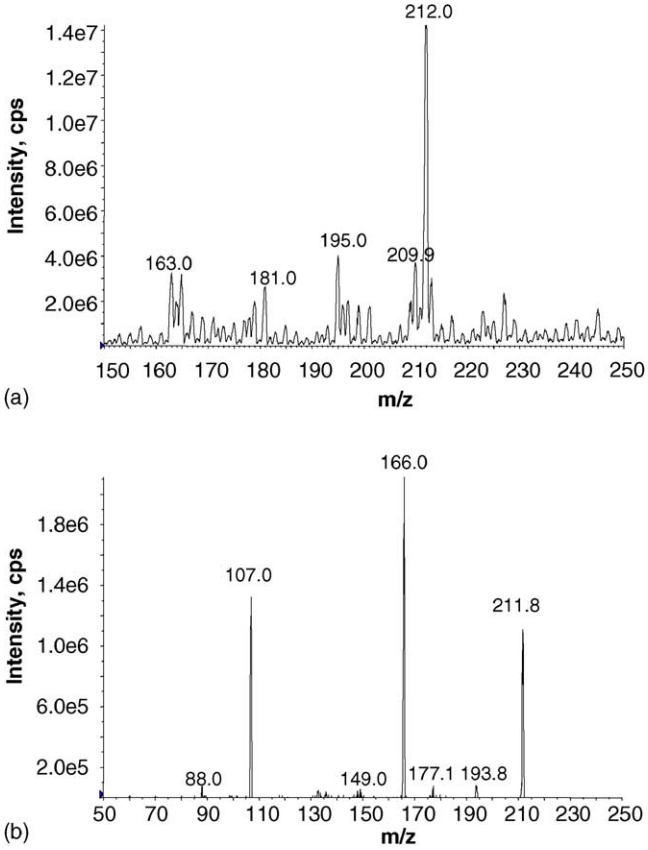

Fig. 3. IS (*m/z* 212) positive ion electrospray mass spectrum (a) and product ion mass spectrum (b).

was 10 L/min, and the ion spray voltage was set to 3000 V. The declustering, focusing, and entrance potentials were set to 26, 150, and 10 V, respectively. Collision gas (nitrogen) flow was maintained at 4 units and the collision energy was set to 20 V. The mass spectrometer quadrupoles were tuned to unit resolution (0.7 Da at 50% height).

Data were acquired with a dwell time of 100 ms for AMT (50 ms for IS), and a scan rate of 6.25 Hz. The longer dwell time was used for AMT to ensure an optimal signal-to-noise ratio for samples with concentrations near the lower limit of quantification, while maintaining an overall scan rate that would result in an adequate number of points acquired to define the chromatographic peaks. The peak area ratios of AMT to IS were plotted versus nominal analyte concentration in order to generate calibration curves. Calibration curves were fitted to a $1/x^2$ quadratic regression using Analyst software (version 1.2).

2.5. Pharmacokinetic Parameters

Data obtained from sample analysis was used to calculate several pharmacokinetic parameters. Elimination half-life ($t_{1/2}$) is the time required for drug plasma concentration to be reduced by half. This parameter is readily determined from a plot of plasma concentration versus time [14]. Total

body clearance (CL/*F*) is the rate at which a drug is removed from the body by all drug elimination processes [15]. Area under the curve (AUC) is the total area under the plasma drug concentration–time curve [16]. Various AUC measurements can be used to determine factors such as drug bioavailability and drug clearance. Volume of distribution (*V*/*F*) is a term used to relate the amount of drug in the body to the measured amount in a biological fluid such as plasma or serum. The apparent volume of distribution during the terminal phase, V_z/F, is calculated by dividing total clearance by a terminal rate constant that is determined from the slope of the time–plasma curve [16].

2.6. Application

Sixteen overtly healthy males participated in the study after providing written informed consent. Health was determined by medical history, physical examination, vital signs, and 12-lead electrocardiogram. Volunteer age ranged from 21 and 50 years, with body mass index (BMI) ranging from 18.5 to 29.9 kg/m^2.

The study protocol was approved by Eli Lilly and Company, and by the Institutional Review Board of National University Hospital, National University of Singapore. The study was conducted at the Lilly-NUS Centre for Clinical Pharmacology, National University of Singapore, in accordance with applicable laws and regulations, good clinical practices, and the ethical principles that have their origin in the Declaration of Helsinki.

The method was used to analyze heparinized plasma samples obtained from healthy human volunteers following a single oral dose of 250 mg AMT. Sequential venous blood samples were collected at 0 (predose), 0.5, 1, 2, 3, 4, 6 and 7 h post-AMT dosing. The plasma samples obtained following centrifugation were kept frozen at −20 °C until analysis.

3. Results and discussion

3.1. Development and validation of the method

Several effective protein precipitation techniques using either organic solvents, acids, or salt and metal ions have been reported [17]. Concerns over the potential effects of organic solvents or acids on ionization suppression and chromatography led to the choice of utilizing 20% zinc sulfate (w/v) in conjunction with 0.5 M NaOH as the precipitant. Absolute recovery (combined extraction efficiency and matrix effect) was determined by comparing peak area responses for AMT and IS from extracted samples with non-extracted samples (no matrix). Absolute AMT and IS recoveries were approximately 75 and 79%, respectively. In addition to being an efficient extraction technique, the extraction procedure produced a nearly 100% aqueous final sample extract solution that could be directly injected into the LC–MS–MS system. This approach resulted in a substantial decrease in sample preparation time by eliminating the need for sample drying and reconstitution steps.

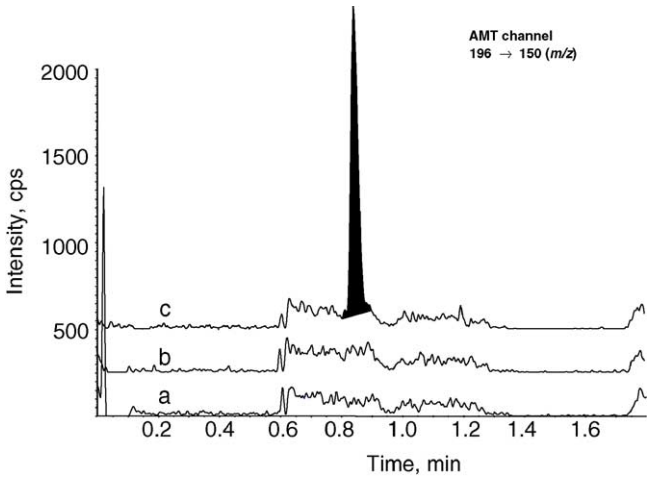

Fig. 4. Representative mass chromatograms for (a) blank human plasma, (b) blank human plasma plus internal standard, and (c) 50 ng/mL LLOQ samples (8.3 pg on column) (chromatograms were offset for illustrative purposes).

Several reverse-phase HPLC columns were evaluated during method development. Some columns such as the MetaChem MonoChrom C_{18}, MacMod Analytical Ace C_{18}, and MetaChem Inertsil ODS-3 displayed acceptable chromatography, while others such as the MacMod Zorbax Analytical SB-C_{18}, Phenomenex Luna Phenyl-Hexyl, and Waters Xterra MS C_{18} were rejected due to excessive analyte peak tailing, or inadequate retention of the compounds. All columns that were evaluated had 2.0 mm × 50 mm dimensions and 5 μm particle size. The MetaChem MonoChrom C_{18} column was chosen because it provided superior results for both AMT and IS.

The selected reaction monitoring $(M + H)^+$ transitions monitored for AMT and IS (*m*/*z* 196 → 150 and *m*/*z* 212 → 166, respectively) were attributed to the neutral loss of the carboxylic acid moiety for both molecules (Figs. 2 and 3). Other product ions were observed, but these transitions were chosen because they displayed the greatest intensity. While multiple transitions could have been monitored during validation to ensure the 'purity' of the signal, it was not deemed necessary for this controlled study involving the administration of a single well-characterized compound. An *m*/*z* 209.9 ion observed in the mass spectra for both AMT and IS was attributed to the polypropylene glycol solution used to tune and calibrate the mass spectrometer. An *m*/*z* 195 ion was present in the IS mass spectrum. Although this ion was only mass resolved by one unit from AMT (*m*/*z* 196), no *m*/*z* 150 ion was observed in the IS product ion spectrum, and no AMT interference peaks were observed in the *m*/*z* 196 → 150 channel for extracted blanks containing only IS (Fig. 4).

Calibration curves were constructed by plotting the AMT/IS peak area ratios for the standards against the known concentrations. Once plotted, the calibration curves were used to determine AMT concentrations in validation, QC,

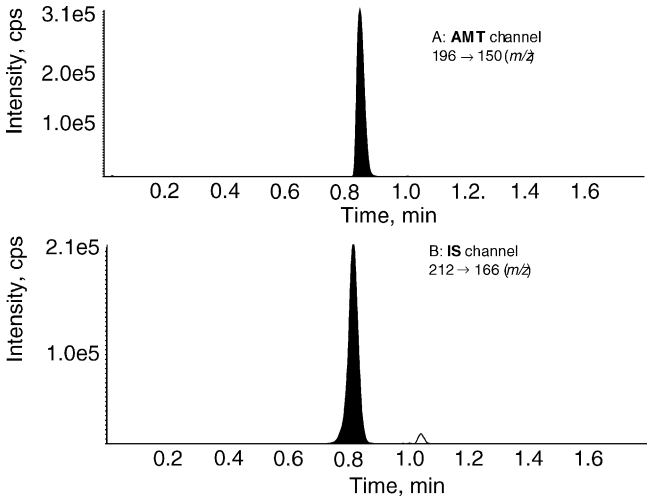

Fig. 5. Representative mass chromatograms for injection of neat solution (water–acetic acid (100:0.1, v/v)) containing AMT and IS (1 ng/mL of each compound on column).

stability and study samples. The calibration curves were fit over the concentration range (50–102,400 ng/mL) using a $1/x^2$ weighted quadratic fit. The correlation coefficient (r) was 0.998 or greater for each of the three validation batches. Standard concentrations were measured for each validation batch and deviated from nominal by less than 15%. Specificity was demonstrated by the absence of interfering peaks at the retention times for AMT and IS in blank samples prepared from five separate human plasma lots (Fig. 4). The retention times for both AMT and IS were approximately 0.8 min (Fig. 5).

Stability samples (n = 3) containing AMT (250 and 89,600 ng/mL) in frozen human plasma were periodically assayed along with fresh standards to determine storage stability. Room temperature stability was determined by allowing stability samples to remain on the bench top for approximately 24 h prior to analysis with fresh standards. Stock solutions were stored at room temperature and compared to fresh stocks in order to determine stability of AMT in neat solution. Stability of AMT in plasma was demonstrated at −20 °C for at least 118 days, and at room temperature for approximately 24 h. Stocks containing AMT at a nominal concentration of 512 μg/mL and stored at room temperature for 14 days were not substantially different (approximately −1%) from freshly prepared stocks. All stability information is summarized as Table 1.

Validation of the method was conducted in accordance to current US Food and Drug Administration guidelines for bioanalytical method validation [18]. The precision of the method was defined as the relative standard deviation (%R.S.D.) calculated from replicate measurements. The accuracy of the assay was defined as the relative error (%RE) of the mean of the replicate measurements from the theoretical values. Precision and accuracy were determined by analyzing validation samples prepared at three concentrations in three separate runs. Six replicates were prepared and analyzed at each concentration. The intra- and inter-day precision and accuracy results are shown as Table 2. All intra- and inter-day precision and accuracy values were acceptable, and spanned the entire concentration range.

Table 2
Validation accuracy and precision summary for α-methyltyrosine in human plasma (n = 3 days, six replicates per day)

Added C (ng/mL)	Found C (ng/mL)	Intra-assay %R.S.D.	Inter-assay %R.S.D.	%RE
50.0	51.6	4.7	6.0	3.3
51200	47567	4.5	5.0	−7.1
102400	99571	5.1	10.2	−2.8

A primary goal during method development was to eliminate sample-to-sample carry-over to the extent that a large dynamic concentration range could be used. Such an approach would eliminate the need to prepare and validate sample dilutions, as well as prevent the need to develop two methods to cover the desired concentration range (50–102,400 ng/mL). Although AMT concentrations for the current study samples did not approach the upper limit of quantitation, this method could readily be used for future studies that incorporate higher patient AMT dosages, if desired. Carry-over was evaluated in each validation batch by injecting a zero standard (blank extracts containing IS) immediately following each of the highest standard samples. Peak area ratios (peak area of AMT/peak area of IS) were calculated for these zero standards and the percent relative carry-over (%RC) was determined by:

$$\%RC = \left[\frac{\text{mean zero standard peak area ratio}}{\text{mean LLOQ peak area ratio}}\right] \times 100$$

for each batch [19]. Carry-over was considered acceptable if %RC was equal to or less than 20%. The %RC was less than 2.5% across the three validation batches.

3.2. Application

The method was successfully used to determine plasma AMT concentrations in healthy human volunteers. For the study presented here, a total of 232 samples were analyzed in two separate batches over 2 days. Representative mass chromatograms from the analysis of plasma samples from subjects dosed with AMT are shown as Fig. 6. Quality control samples were analyzed with each batch, and the intra- and inter-day precision and accuracy results are shown as Table 3. A plasma concentration versus time profile of AMT

Table 3
Quality control sample summary for routine analysis of α-methyltyrosine in human plasma (n = 2 days, four replicates per day)

Added C (ng/mL)	Found C (ng/mL)	Intra-assay %R.S.D.	Inter-assay %R.S.D.	%RE
250	249.8	8.0	7.8	−0.1
51200	49479	10.4	10.0	−3.4
89600	94130	11.6	10.5	5.1

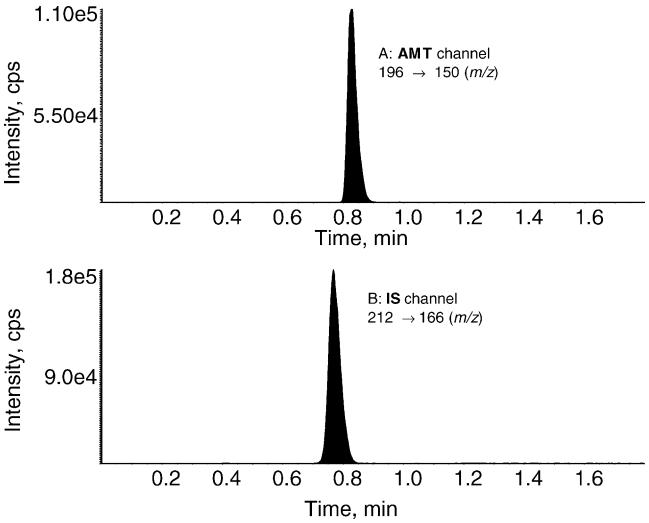

Fig. 6. Mass chromatograms from the analysis of a plasma sample from a subject dosed with AMT.

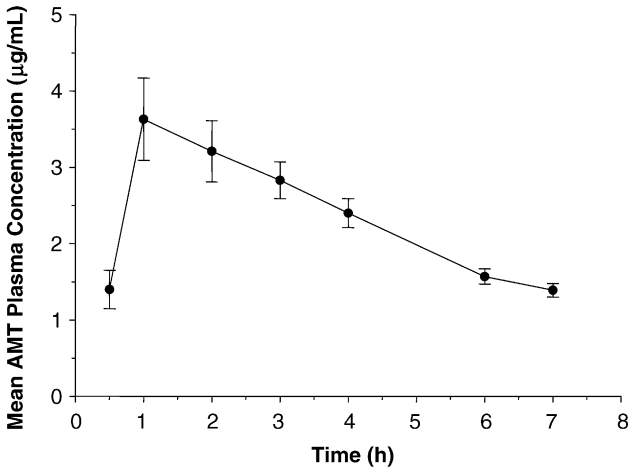

Fig. 7. Representative mean plasma AMT concentration time profile after oral administration of 250 mg AMT in humans (n = 16).

in human plasma is shown in Fig. 7. These data were and could be used to develop a PK/PD model between plasma AMT concentrations and AMT induced prolactin increase in humans. Additionally, the method could be readily applied to the analysis of AMT in other biological matrices (Table 4).

In summary, a novel, sensitive and rapid LC–MS–MS method for isolating and quantifying AMT from human plasma has been developed and validated over the concentration range 50–102,400 ng/mL. The method was precise, accurate, robust, and amenable to high sample throughput. The method has been successfully implemented to derive pharmacokinetic parameters to develop a PK/PD model relating AMT plasma concentrations to prolactin levels.

Table 4
Pharmacokinetic parameters

Parameter	Human (oral, 250 mg, n = 15)
$t_{1/2}$ (h)	3.33 (17.0)
AUC[a] (0–∞) (μg h/mL)	22.5 (22.5)
CL/F[b] (L/h)	11.1 (22.5)
V_z/F[c] (L)	53.4 (24.5)

All values are expressed as the geometric mean (geometric %CV). Descriptive statistics calculated with n = 15, due to the limited pharmacokinetic parameters derived from the data of one subject.

[a] Area under the concentration–time curve from dosing to infinity.

[b] Apparent total body clearance.

[c] Apparent volume of distribution during the terminal phase.

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