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Simultaneous determination of apatinib and its four major metabolites in human plasma using liquid chromatography–tandem mass spectrometry and its application to a pharmacokinetic study

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ABSTRACT

Apatinib, also known as YN968D1, is a novel antiangiogenic agent that selectively inhibits vascular endothelial growth factor receptor-2. Currently, apatinib is undergoing phase II/III clinical trials in China for the treatment of solid tumors. Apatinib is extensively metabolized in humans, and its major metabolites in circulation include *cis*-3-hydroxy-apatinib (M1-1), *trans*-3-hydroxy-apatinib (M1-2), apatinib-25-*N*-oxide (M1-6), and *cis*-3-hydroxy-apatinib-*O*-glucuronide (M9-2). To investigate the pharmacokinetics of apatinib and its four major metabolites in patients with advanced colorectal cancer, a sensitive and selective liquid chromatography–tandem mass spectrometry method was developed and validated for the simultaneous determination of apatinib, M1-1, M1-2, M1-6, and M9-2 in human plasma. After a simple protein precipitation using acetonitrile as the precipitation solvent, all the analytes and the internal standard vatalanib were separated on a Zorbax Eclipse XDB C_{18} column (50 mm × 4.6 mm, 1.8 μm, Agilent) using acetonitrile: 5 mmol/L ammonium acetate with 0.1% formic acid as the mobile phase with gradient elution. A chromatographic total run time of 9 min was achieved. Mass spectrometry detection was conducted through electrospray ionization in positive ion multiple reaction monitoring modes. The method was linear over the concentration range of 3.00–2000 ng/mL for each analyte. The lower limit of quantification for each analyte was 3.00 ng/mL. The intra-assay precision for all the analytes was less than 11.3%, the inter-assay precision was less than 13.8%, and the accuracy was between −5.8% and 3.3%. The validated method was successfully applied to a clinical pharmacokinetic study following oral administration of 500 mg apatinib mesylate in patients with advanced colorectal cancer.

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1. Introduction

Angiogenesis is an essential step in tumor growth and metastasis. Vascular endothelial growth factor receptors (VEGFRs) are tyrosine kinases functioning as key regulators of this process. The VEGFR-family proteins consist of VEGFR-1 (Flt-1), VEGFR-2 (KDR/Flk-1), and VEGFR-3 (Flt-4). Among these receptors, VEGFR-2 is thought to be principally responsible for angiogenesis in malignancies [1].

Apatinib (*N*-[4-(1-cyano-cyclopentyl)phenyl]-2-(4-pyridylmethyl) amino-3-pyridine carboxamide), also known as YN968D1, is a novel orally bioavailable small molecule antiangiogenic agent that selectively inhibits VEGFR-2 and also mildly inhibits c-Kit and c-SRC tyrosine kinases [2]. Apatinib was first synthesized by Advenchen Laboratories in California, USA and is currently being developed by Jiangsu Hengrui Medicine, China. The vatalanib (PTK787)-derived compound apatinib demonstrates superior in vivo efficacy compared with vatalanib [3]. It is currently undergoing phase II/III clinical trials in China for the treatment of many cancer types, such as metastatic gastric carcinoma, metastatic breast cancer, advanced hepatocellular carcinoma, and advanced colorectal cancer. These clinical trials demonstrate that apatinib has potential antitumor activity across a broad range of advanced solid tumors.

A published report on the safety and pharmacokinetics of apatinib in human clinical studies showed that apatinib can be well tolerated and exhibits substantial antitumor activity at a dose of 750 mg once daily [4]. Apatinib demonstrated quick absorption, with C_{max} reached in 3–4 h. The mean half-life was estimated to be approximately 9 h and constant over all dose groups. C_{max} values varied between 926 and 4625 ng/mL after a single dose of 750 mg, which showed high inter-patient variability. Steady-state conditions were achieved within 6 d of dosing, with no accumulation during 56 d of once daily dosing of apatinib. However, this report neither mentioned the pharmacokinetics of the metabolites of apatinib nor provided detailed descriptions of the analytical method used in the pharmacokinetic study.

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Table 1
MS parameters for apatinib, M1-1, M1-2, M1-6, M9-2, and vatalanib (IS).

Compound	Precursor ion $[M+H]^+$ (*m*/*z*)	Product ion (*m*/*z*)	Collision energy (eV)	Declustering potential (V)
Apatinib	398	212	26	80
M1-1	414	212	31	80
M1-2	414	212	31	80
M1-6	414	108	30	100
M9-2	590	414	33	100
Vatalanib	347	311	46	90

The metabolism of apatinib in humans was studied in our laboratory (data not shown). Apatinib is extensively metabolized in humans via oxidative (phase I) and conjugative (phase II) pathways. Based on the peak area ratios of the metabolites to the parent drug, the main metabolites of apatinib in human plasma include *cis*-3-hydroxy-apatinib (M1-1), *trans*-3-hydroxy-apatinib (M1-2), apatinib-25-*N*-oxide (M1-6), and *cis*-3-hydroxy-apatinib-*O*-glucuronide (M9-2). In the present study, a sensitive and selective chromatography–tandem mass spectrometry (LC–MS/MS) method was developed and validated for the simultaneous quantification of apatinib, M1-1, M1-2, M1-6, and M9-2 in human plasma. The validated methods were successfully applied to characterize the pharmacokinetic profiles of apatinib and its four major metabolites following oral administration of 500 mg apatinib mesylate once daily for 28 d in patients with advanced colorectal cancer.

2. Experimental

2.1. Materials

Reference standards of apatinib mesylate (99.74% purity), M1-1 (94.75% purity), M1-2 (96.76% purity), and vatalanib (99.8% purity, internal standard, IS) were provided by Jiangsu Hengrui Medicine Co., Ltd. (Lianyungang, China). Reference standard of M1-6 (100.0% purity) was synthesized and purified in our laboratory. Reference standard of M9-2 (100.0% purity) was isolated and purified from human urine in our laboratory. Chemical structures of these reference standards were confirmed through electrospray ionization tandem mass spectrometry (ESI-MS/MS) and ^{1}H nuclear magnetic resonance (^{1}H NMR) analysis. HPLC-grade methanol, acetonitrile, and formic acid were purchased from Sigma–Aldrich (St. Louis, MO, USA). HPLC-grade ammonium acetate was purchased from ROE (Newmark, DE, USA). Analytical grade of dimethyl sulfoxide (DMSO) was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Deionized water was purified by a Millipore Milli-Q Gradient Water Purification System (Molsheim, France).

2.2. Instrumentation

A Shimadzu HPLC system consisting of a DGU-20A$_3$ vacuum degasser, a LC-20AD binary pump, a CTO-20A column oven, and a SIL-20AC autosampler (Shimadzu, Kyoto, Japan) was used for solvent and sample delivery. An API 4000 triple quadrupole mass spectrometer (Applied Biosystems, Concord, Ontario, Canada) equipped with a Turbo IonSpray ionization (ESI) source was used for mass analysis and detection. Data acquisition and processing were performed using Analyst 1.4.2 software (Applied Biosystems).

2.3. LC–MS/MS analytical conditions

Apatinib, M1-1, M1-2, M1-6, M9-2, and vatalanib (IS) were separated under gradient elution using a Zorbax Eclipse XDB-C$_{18}$ column (50 mm × 4.6 mm internal diameter, 1.8 μm particle size, Agilent, Santa Clara, CA, USA) with a C$_{18}$ guard column (4.0 mm × 3.0 mm internal diameter, 5 μm particle size, Phenomenex, Torrance, CA, USA). The column oven was set at room temperature. The mobile phase consisted of solvent A (5 mmol/L ammonium acetate solution with 0.1% formic acid) and solvent B (acetonitrile). The initial condition was 78% solvent A and 22% solvent B from 0 min to 3 min. A linear gradient was performed, with mobile phase B increasing from 22% to 80% within 1 min and held for 2 min. The mobile phase was returned to the initial condition and re-equilibrated for 3 min. The total analysis time was 9 min. The flow rate was set at 0.5 mL/min. The injection volume was 10 μL.

The mass spectrometer was operated in a positive ion mode with nebulizer gas (Gas 1), heater gas (Gas 2), curtain gas, and collision gas (all nitrogen) at the flow rates of 50, 50, 30, and 6 psi, respectively. The ion spray voltage was 4200 V, and the source temperature was 400 °C. The dwell time was kept at 150 ms for each transition. A summary of the optimized multiple reaction monitoring (MRM) fragmentation transitions and MS parameters for each analyte is reported in Table 1.

2.4. Preparation of calibration standards and quality control (QC) samples

The calibration standards and QC samples were prepared from two separate sets of solutions parallel to each other. Stock solutions of apatinib, M1-1, M1-2, and vatalanib (IS) at 1.00 mg/mL were prepared individually by dissolving the accurately weighted reference substances in a small amount of DMSO and methanol. Stock solutions of M1-6 and M9-2 at 1.00 mg/mL were prepared by dissolving the accurately weighed reference substances in methanol. The concentration of each analyte was calculated as free base. A standard working solution containing 100 μg/mL of apatinib, M1-1, M1-2, M1-6, and M9-2 was prepared by appropriately mixing the five stock solutions and diluting with 50% aqueous methanol. The obtained mix solution was then serially diluted with 50% aqueous methanol to achieve standard working solutions at the following concentrations: 3.00, 10.0, 30.0, 100, 300, 1000, and 2000 ng/mL for each analyte, giving a calibration range of 3.00–2000 ng/mL for each analyte. The lower limit of quantification (LLOQ) samples (at a concentration of 3.00 ng/mL for each analyte) and QC samples (at concentrations of 6.00, 80.0, and 1600 ng/mL for each analyte) were independently and similarly prepared in blank plasma. An IS working solution (500 ng/mL) was prepared by diluting the vatalanib stock solution (1.00 mg/mL) with 50% aqueous methanol. All solutions were kept refrigerated (4 °C) and brought to room temperature before use. The QC samples were dispatched in 500 μL aliquots and stored in plastic tubes at −20 °C until analysis.

2.5. Sample preparation

The frozen plasma samples were thawed at room temperature and vortexed thoroughly. To a 50 μL aliquot of plasma sample, 50 μL of IS solution (500 ng/mL vatalanib), 50 μL of 50% aqueous methanol, and 200 μL of acetonitrile were added. The mixture was vortex-mixed for 1 min and centrifuged at 11,600 × g for 5 min. A 50 μL aliquot of the supernatant liquid was transferred into another tube and mixed with 100 μL of 5 mmol/L ammonium acetate solution with 0.1% formic acid. A 10 μL aliquot of the

resulting solution was injected onto the LC–MS/MS system for analysis.

2.6. Method validation

A validation of the assay was performed according to the FDA guideline for validation of bioanalytical assays including selectivity, linearity, precision and accuracy, recovery, matrix effect and stability [5].

To investigate the selectivity of the method, six blank plasma samples and twelve spiked plasma samples at the LLOQ level from six different donors were analyzed. The MRM chromatograms of the blank plasma samples were compared with those of the corresponding spiked plasma samples at the LLOQ level. The peak area of coeluting interferences should be less than 20% of the peak area of the analytes and less than 5% of the peak area of IS.

To evaluate linearity, the calibration curves were prepared and analyzed in duplicate in three independent days. Linearity was assessed by plotting the peak area ratios (analyte/IS) versus the corresponding nominal concentrations using a linearly weighted ($1/x^2$) least squares regression. A correlation coefficient (r^2) greater than 0.990 was required for determination of linearity. The deviations of the calculated concentrations should be within ±15% of the nominal concentrations except for the LLOQ, at which a deviation of ±20% is permitted.

Precision and accuracy were assessed by determining the concentrations of QC samples at three levels (low, 6.00 ng/mL; medium, 80.0 ng/mL; high, 1600 ng/mL) using six replicates on three consecutive validation days. Precision was expressed as the intra- and inter-day relative standard deviation (RSD). Accuracy was expressed as the relative error (RE) between the mean measured concentration and the nominal concentration. For acceptance, the intra- and inter-day precisions were required not to exceed 15%, and accuracy should be within ±15%.

The LLOQ, defined as the lowest concentration on the calibration curve with an acceptable precision (RSD not exceeding 20%) and accuracy (RE within ±20%), was evaluated by analyzing the samples prepared in six replicates on three consecutive days.

Dilution tests with blank matrix for the method were performed. Validation samples with analyte concentrations above the upper limit of quantification (ULOQ) were diluted 1:10 (v/v) prior to analysis by blank human plasma to be within the validated range. These samples were processed in six replicates and measured in a single analytical run with a dilution factor of 1:10 to assess the accuracy and precision after sample dilution.

The recovery of all the analytes was estimated at three QC levels (6.00, 80.0, and 1600 ng/mL for each analyte) by comparing the mean peak areas of the QC samples ($n = 6$) with those of the blank plasma samples spiked with neat solutions after extraction ($n = 3$). The recovery of IS was determined in a similar way at 500 ng/mL.

The matrix effect was quantitatively measured as IS-normalized matrix factor at concentrations of 6.00 and 1600 ng/mL for each analyte by dividing the peak area ratios (analyte/IS) in extracted samples of blank plasma from six subjects spiked with the analytes and IS by the corresponding peak area ratios (analyte/IS) in water-substituted samples at equivalent concentrations. The matrix effect of IS was determined in a similar way at 500 ng/mL. Inter-subject variability of the IS-normalized matrix factors, as measured by the RSD should be less than 15% [6].

Stability was examined in plasma samples, stock solutions, and working solutions under different conditions (time and temperature). For the stability study of plasma samples, all the analytes were investigated by analyzing replicates ($n = 3$) of plasma samples at two concentrations (6.00 and 1600 ng/mL for each analyte). The long-term stability was evaluated after storage of the plasma samples at −20 °C for 47 d. The short-term stability was assessed after the exposure of the spiked samples at room temperature for 6 h. The post-preparative stability was measured after exposure of processed samples at room temperature for 48 h. The freeze/thaw stability was determined after three freeze/thaw cycles (−20 °C to room temperature) of the spiked samples on consecutive days. The analytes were considered stable when their RE was within ±15% of the initial concentration. For the stability study of stock solutions, stock solutions of all the analytes and IS were checked for short-term stability after 6 h of storage at room temperature and for long-term stability after 48 d at 4 °C. For the stability study of working solutions, standard solution at concentration of LLOQ (3.00 ng/mL for each analyte) and working solution of IS (500 ng/mL) were checked for long-term stability after 28 d at 4 °C. Solutions were deemed stable if the peak area difference between the stored solution and a freshly prepared solution is ≤10%.

2.7. Assay application to a clinical pharmacokinetic study

The validated LC–MS/MS assay was applied to investigate the plasma profiles of apatinib and its four major metabolites following oral administration of 500 mg apatinib mesylate tablets (Jiangsu Hengrui Medicine Co., Ltd., Lianyungang, China) once daily for 28 d in five (three males and two females) patients with advanced colorectal cancer. The pharmacokinetic study was approved by the Ethics Committee of Fudan University Shanghai Cancer Center (Shanghai, China), conducted in accordance with the Declaration of Helsinki and the principles of Good Clinical Practice. Written informed consents were obtained from all subjects prior to enrollment. Blood samples (2–3 mL) were collected into sodium heparin containing tubes at 0 (predose), 0.5, 1, 2, 3, 4, 6, 8, 12, and 24 h (before the next dose) on day 1 and day 28 and centrifuged at 1000 × *g* for 10 min to separate the plasma fractions. The plasma samples were stored at −20 °C until analysis.

2.8. Incurred sample reanalysis (ISR)

The concept of ISR was established in the third AAPS/FDA bioanalytical workshop/conference report [7]. As stated in this conference, the performance of spiked standards and QCs may not adequately mimic that of study samples from dosed subjects, that is, incurred samples. Thus, a proper evaluation of incurred sample reproducibility and accuracy need to be performed. The ISR program has recently been accepted and commonly practiced in the pharmaceutical industry and bioanalytical laboratories as an additional measure to evaluate the reliability of the data and assay performance. The number of samples repeated for ISR analysis is recommended to be 5–10% of the total sample size. It is recommended that ISR study samples be obtained from several subjects close to the expected maximal concentration and near the end of the elimination phase. The difference between the two values obtained should be within 20% of the mean for at least 67% of the repeats.

Reproducibility of the analytical method was further evaluated through ISR. About 400 plasma samples were analyzed for the apatinib clinical trials in this study, of which 20 samples were selected for ISR to further evaluate the reproducibility of the analytical method. The ISR data were compared with the data from the original assay.

3. Results and discussion

3.1. Mass spectrometry

For the optimization of MS parameters, analyte solutions (concentration of 100 ng/mL) were directly infused into the ion source of the mass spectrometer through a syringe pump operating at a speed of 20 μL/min.

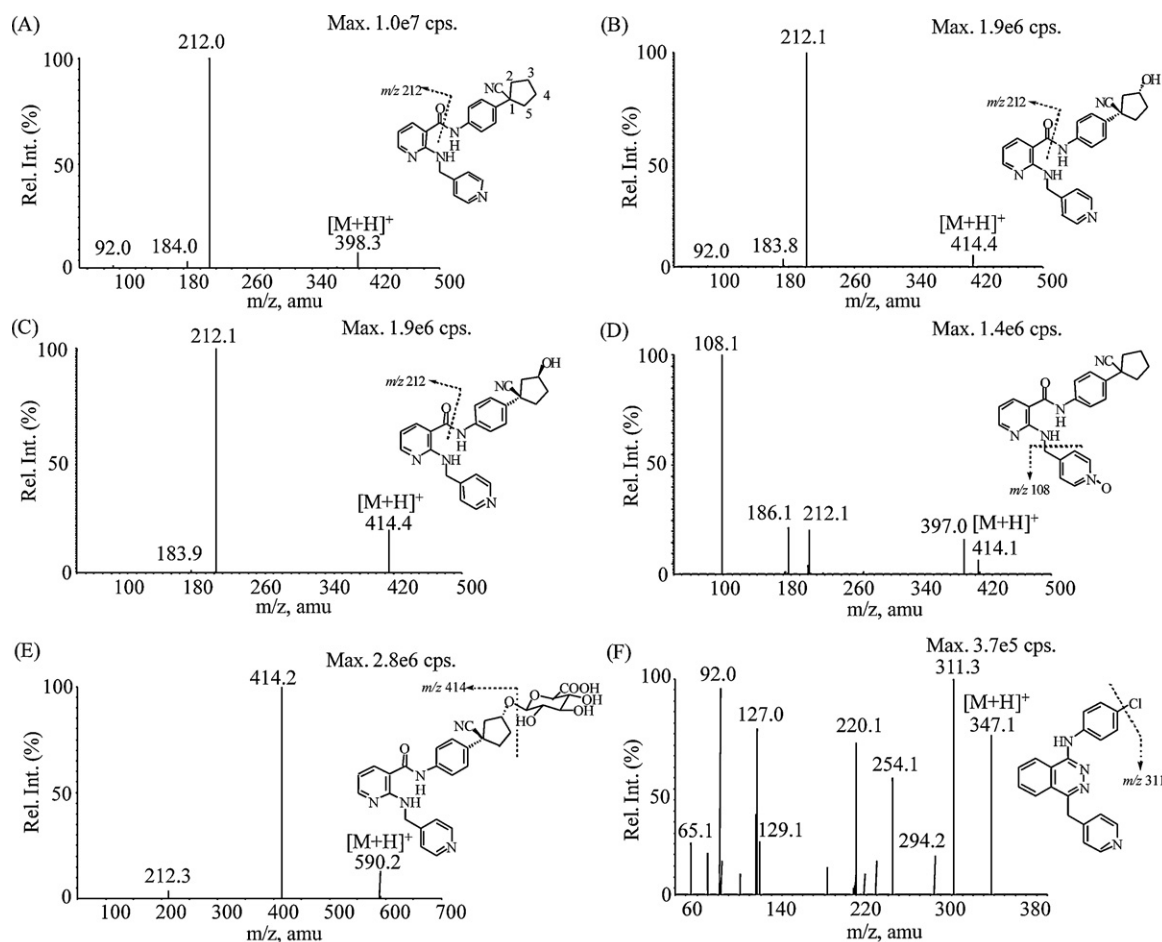

Fig. 1. Product ion spectra of [M+H]+ of apatinib (A), M1-1 (B), M1-2 (C), M1-6 (D), M9-2 (E), and the internal standard vatalanib (F) and their proposed fragmentation patterns.

Apatinib, M1-1, M1-2, M1-6, M9-2, and IS all have basic secondary and tertiary nitrogen groups in their structures. Consequently, they exhibited much higher MS responses under a positive ion detection mode. The ESI source resulted in much higher efficiency for ionization of the target analytes than the atmospheric pressure ionization source. Thus, we selected ESI as the ionization mode for this study.

In the Q1 full scan mode, protonated molecules at *m/z* 398, *m/z* 414, *m/z* 414, *m/z* 414, *m/z* 590, and *m/z* 347 were observed for apatinib, M1-1, M1-2, M1-6, M9-2, and IS, respectively. Fig. 1 presents the product ion spectra of [M+H]+ ions from the analytes and IS, as well as their proposed fragmentation patterns. The most abundant and stable fragment ions at *m/z* 212, 212, 212, 108, 414, and 311 were selected in the MRM transitions for apatinib, M1-1, M1-2, M1-6, M9-2, and IS, respectively.

The highest MS response of apatinib (*m/z* 398 → *m/z* 212), M1-1 (*m/z* 414 → *m/z* 212), and M1-2 (*m/z* 414 → *m/z* 212) was achieved when the CE value was set at 36, 38, and 38 eV, respectively. However, the MS response of apatinib, M1-1, and M1-2 tended to be saturated when high concentrations of calibration standards were analyzed. Therefore, the CE value of apatinib, M1-1, and M1-2 was finally set at 26, 31, and 31 eV to achieve proper MS responses.

3.2. Chromatography

Apatinib has six mono oxygenated metabolites in human plasma (data not shown). Among these metabolites, only M1-1, M1-2, and M1-6 were selected to be determined in this study because the concentrations of the others were very low compared with the parent drug. The six mono oxygenated metabolites were indistinguishable via MS. Both M1-1 and M1-2 underwent glucuronidation in humans, but the concentration of M9-2 (the glucuronide of M1-1) was much higher than that of M9-1 (the glucuronide of M1-2) in circulation. Therefore, we only determined one of the isomers (M9-2) in human plasma. M9-2 and M9-1 were also indistinguishable via MS. Both M9-2 and M9-1 can be converted back to their corresponding aglycon because of in-source collision-induced dissociation in MS. Consequently, the chromatographic separation of all mono oxygenated metabolites, M9-1 and M9-2 should be achieved. The in-source collision-induced dissociation of M1-6 (the *N*-oxide metabolite of apatinib) to break down to the parent drug was negligible.

Various chromatographic conditions of different HPLC columns and mobile phase compositions were tested to retain all the analytes and baseline separate all mono oxygenated metabolites, M9-1, and M9-2 without requiring long run times. The log *P* values of apatinib, M1-1, M1-2, M1-6, M9-2, and IS are 3.68, 2.05, 2.05, 1.75, 0.01, and 3.80, respectively (calculated using Advanced Chemistry Development software, ADME Suite, Version 5.0). Isomers M1-1 and M1-2 have the same polarity, while the others have considerably different polarities. As a result, isocratic elution could not meet the requirements of the simultaneous detection, and gradient elution was selected to obtain a better resolution and maintain the retention of all the analytes in a short chromatographic time. Many HPLC columns including Capcell C_{18}, Gemini C_{18}, Atlantis C_{18}, and XDB C_{18} were evaluated in terms of peak shape and separation

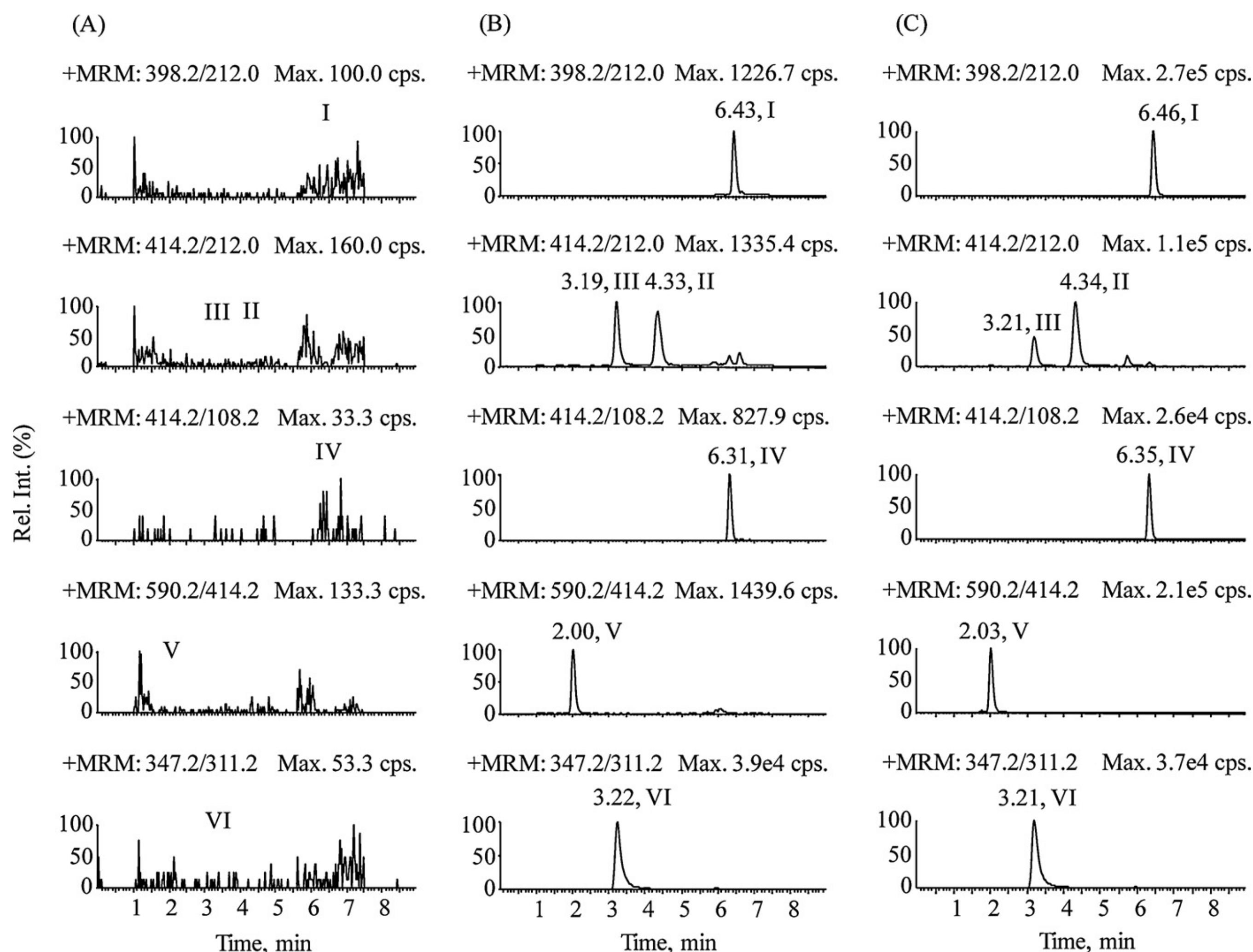

Fig. 2. Representative MRM chromatograms of apatinib (I), M1-1 (II), M1-2 (III), M1-6 (IV), M9-2 (V), and vatalanib (IS, VI) in human plasma: (A) blank plasma sample, (B) plasma spiked with 3.00 ng/mL of each analyte and 500 ng/mL vatalanib (IS), and (C) plasma sample 4 h after oral administration of 500 mg of apatinib mesylate once daily for the first day.

capacity. The result showed that only the Zorbax Eclipse XDB-C_{18} column (50 mm × 4.6 mm internal diameter, 1.8 μm particle size) performed satisfied peak separation and peak symmetry for each analyte. Thus, it was selected for further evaluation. The use of ammonium acetate as a buffer in the mobile phase improved the peak symmetry and reproducibility of retention time, and the use of formic acid in the mobile phase improved the signal and chromatography of the analytes. Thus, the mobile phase was finally optimized as acetonitrile: 5 mmol/L ammonium acetate solution with 0.1% formic acid using a gradient elution. Vatalanib, an analog of apatinib, was readily available and selected as the IS. It produced a good peak shape and suitable retention time on the HPLC column. Under the chromatographic conditions employed, the retention times were 6.4, 4.3, 3.2, 6.3, 2.0, and 3.2 min for apatinib, M1-1, M1-2, M1-6, M9-2, and IS, respectively (Fig. 2).

3.3. Sample preparation

Considering the large batches of plasma samples during the clinical trials, a simple sample preparation procedure was required. Protein precipitation (PPT) and single step liquid–liquid extraction (LLE) were primary considered for their simplicity and efficiency. PPT is well known as a nonselective purification method that may introduce high amounts of endogenous components and can cause signal suppression or enhancement especially with an ESI ionization source. Therefore, our first attempt was to develop an LLE procedure. Extraction with ethyl acetate or ethyl ether–dichloromethane (2:1, v/v) after pH modification of plasma with a 0.1 mol/L sodium hydroxide or a phosphate buffer at pH 7 or 10 (200 μL of plasma and 200 μL of buffer) was performed. At any pH level, M9-2 recovery did not exceed approximately 10%. PPT was tested because LLE did not permit extraction of all the analytes in a single step. The results showed that the matrix effect was negligible at the retention time of each analyte. Therefore, PPT by acetonitrile yielded consistent and high recovery rates without matrix interference and was employed.

3.4. Validation

3.4.1. Selectivity

Fig. 2 shows the typical chromatograms of blank plasma, plasma spiked with apatinib, M1-1, M1-2, M1-6, and M9-2 at the LLOQ (3.00 ng/mL for each analyte) and IS (500 ng/mL), and a plasma sample obtained 4 h after oral administration of 500 mg apatinib mesylate once daily for the first day to a patient with advanced colorectal cancer. No interfering peaks from endogenous compounds were observed at the retention time of all the analytes and IS.

Table 2
Precision and accuracy data for analysis of apatinib, M1-1, M1-2, M1-6, and M9-2 in human plasma (3 d with 6 replicates per day).

Analyte	Concentration (ng/mL)		RSD (%)		RE (%)
	Spiked	Calculated	Intra-day	Inter-day	
Apatinib	3.00	3.23	9.3	12.1	7.8
	6.00	5.79	10.7	11.7	−3.4
	80.0	83.4	5.4	7.2	4.3
	1600	1532	2.9	7.2	−4.3
M1-1	3.00	2.88	6.8	8.1	−3.8
	6.00	6.04	5.7	11.6	0.7
	80.0	82.0	5.5	7.8	2.5
	1600	1653	2.8	9.7	3.3
M1-2	3.00	2.94	8.0	3.8	−1.8
	6.00	6.07	5.1	11.3	1.2
	80.0	82.5	4.8	4.2	3.1
	1600	1634	2.7	9.6	2.1
M1-6	3.00	3.03	11.3	9.0	0.9
	6.00	5.65	6.0	7.1	−5.8
	80.0	81.7	5.7	5.8	2.2
	1600	1609	3.9	12.7	0.6
M9-2	3.00	3.06	9.4	13.0	2.0
	6.00	5.93	8.4	13.8	−1.1
	80.0	82.1	6.6	5.0	2.6
	1600	1611	3.6	6.9	0.7

3.4.2. Linearity of calibration curve and lower limit of quantification

The calibration curve was linear over the concentration range of 3.00–2000 ng/mL for each analyte. The mean ± standard deviation (SD) linear regression equations of the calibration curves generated during the validation were as follows:

Apatinib: $y = (0.00449 \pm 0.00010)\ x + (0.00935 \pm 0.00274)$ $(r = 0.9968 \pm 0.0016)$;
M1-1: $y = (0.0105 \pm 0.0003)\ x + (0.00288 \pm 0.00128)$ $(r = 0.9980 \pm 0.0006)$;
M1-2: $y = (0.00905 \pm 0.00031)\ x + (0.00128 \pm 0.00030)$ $(r = 0.9978 \pm 0.0004)$;
M1-6: $y = (0.00377 \pm 0.00001)\ x + (0.00309 \pm 0.00117)$ $(r = 0.9981 \pm 0.0010)$;
M9-2: $y = (0.00587 \pm 0.00015)\ x + (0.000781 \pm 0.001160)$ $(r = 0.9970 \pm 0.0010)$

where y represents the peak area ratio of each analyte to the IS, and x is the nominal concentration of the analyte.

The LLOQ was 3.00 ng/mL for all the analytes, with acceptable accuracy and precision (Table 2). The present LLOQ was sensitive enough to allow for the investigation of the pharmacokinetic behavior of apatinib and its four metabolites following oral administration of apatinib mesylate.

3.4.3. Precision and accuracy

Intra- and inter-day precision and accuracy values for the QC samples are summarized in Table 2. The intra-assay precision for all the analytes was less than 11.3%, the inter-assay precision was less than 13.8%, and the accuracy was between −5.8% and 3.3%. In this assay, the intra- and inter-day precision and accuracy values were within the acceptable range. Thus, the method was judged to be reliable and reproducible.

3.4.4. Dilution by blank matrix

The ability to dilute samples containing apatinib, M1-1, M1-2, M1-6, and M9-2 at concentrations above the ULOQ was demonstrated by performing a 10-fold dilution of human plasma validation sample spiked at 10,000 ng/mL of each analyte with blank human plasma. These samples were processed in six replicates and measured in a single analytical run with a dilution factor of 1:10 to assess the accuracy and precision after sample dilution. The results indicated that a 10-fold dilution of human plasma samples containing all the analytes above the ULOQ is acceptable (RSD ≤4.5%, RE in the range of −5.1% to −2.6% for all the analytes).

3.4.5. Recovery

The recoveries ranged from 90.0% to 102% for all the analytes across the concentration range (Table 3). The recovery of IS at 500 ng/mL was 96.2%. The precision (% RSD) values of recoveries for all the analytes and IS were lower than 10.0%. The recoveries were consistent and reproducible.

3.4.6. Matrix effect

The matrix effects ranged from 96.3% to 104% for all the analytes across the concentration range (Table 3). The matrix effect of IS at 500 ng/mL was 101%. Inter-subject variability of the IS-normalized matrix factors, as measured by the RSD was lower than 8.5%. The results showed that ion suppression or enhancement from the plasma matrix was negligible under the current conditions.

3.4.7. Stability

The stability data of spiked plasma and extracts obtained are shown in Table 4. Based on the results, the analytes were stable in plasma stored at room temperature for 6 h, in plasma after three freeze/thaw cycles (−20 °C to room temperature) on consecutive days, in plasma stored at −20 °C for 47 d, and in plasma extracts stored at room temperature for 48 h.

A summary of assay stability of stock solutions and working solutions is presented in Table 5. The stock solutions of apatinib, M1-1, M1-2, M1-6, M9-2, and IS were stable for 6 h at room temperature and under refrigeration at 4 °C for up to 48 d. The standard solution and IS working solution were stable for 28 d at 4 °C.

The results showed that each analyte had an acceptable stability under the test conditions.

3.5. Method application

This validated method was successfully applied in the determination of the plasma concentrations of apatinib, M1-1, M1-2, M1-6, and M9-2 in patients with advanced colorectal cancer

Table 3
Matrix effect and recovery of apatinib, M1-1, M1-2, M1-6, M9-2 and vatalanib (IS) ($n = 6$).

Analyte	Concentration (ng/mL)	Recovery		Matrix effect	
		Mean (%)	RSD (%)	Mean (%)	RSD (%)
Apatinib	6.00	97.9	6.0	98.8	5.8
	80.0	99.9	5.2		
	1600	98.1	3.1	102	1.8
M1-1	6.00	90.2	4.3	96.3	4.2
	80.0	98.1	1.8		
	1600	96.7	1.7	99.2	1.6
M1-2	6.00	94.1	4.9	97.7	4.0
	80.0	98.2	2.2		
	1600	97.2	1.4	99.6	2.1
M1-6	6.00	92.7	7.1	104	4.6
	80.0	90.0	2.8		
	1600	90.0	4.1	102	1.7
M9-2	6.00	94.3	10.0	96.7	8.5
	80.0	102	6.1		
	1600	101	3.0	99.8	3.4
IS	500	96.2	3.3	101	6.0

Table 4
Stability of apatinib, M1-1, M1-2, M1-6 and M9-2 in human plasma under various storage conditions ($n = 3$).

Analyte	Concentration spiked (ng/mL)	Long-term (−20 °C 47 d)		Short-term (room temperature 6 h)		Post-preparative (room temperature 48 h)		3 Freeze–thaw (−20 °C to room temperature)	
		RSD (%)	RE (%)	RSD (%)	RE (%)	RSD (%)	RE (%)	RSD (%)	RE (%)
Apatinib	6.00	7.7	7.9	3.0	0.5	7.2	4.9	13.1	1.4
	1600	2.7	1.3	2.5	−1.8	7.8	−1.1	1.2	−0.9
M1-1	6.00	5.2	−1.0	1.5	0.4	9.2	−1.0	3.2	3.6
	1600	1.3	5.3	3.4	1.2	2.8	4.9	3.5	5.9
M1-2	6.00	2.1	2.1	2.1	7.5	2.8	−0.4	3.1	2.3
	1600	2.3	5.7	1.2	0.3	2.6	2.8	0.3	5.1
M1-6	6.00	7.7	1.9	4.3	6.0	2.9	3.2	2.8	−4.9
	1600	3.3	3.6	3.0	9.2	3.2	6.9	1.5	3.5
M9-2	6.00	4.3	2.3	8.0	2.5	12.5	5.3	6.1	−4.3
	1600	3.3	6.8	0.6	−3.5	2.7	−2.4	1.6	3.6

Table 5
Stability of apatinib, M1-1, M1-2, M1-6, M9-2 and vatalanib (IS) in stock solutions and working solutions under various storage conditions ($n = 6$).

Storage condition	Analyte	RSD (%)		RE (%)
		Freshly prepared	After storage	
Stock solutions at room temperature, 6 h	Apatinib	5.2	2.4	4.7
	M1-1	1.7	1.7	2.2
	M1-2	3.7	1.8	4.2
	M1-6	3.4	3.1	1.1
	M9-2	3.7	3.0	−0.5
	IS	3.0	2.0	−2.0
Stock solutions at 4 °C, 48 d	Apatinib	5.2	2.6	4.1
	M1-1	1.7	1.6	2.2
	M1-2	3.7	2.3	4.6
	M1-6	3.4	2.0	0.9
	M9-2	3.7	2.6	−2.0
	IS	3.0	4.2	0.9
LLOQ solution at 4 °C, 28 d	Apatinib	2.2	1.1	−3.1
	M1-1	1.8	1.5	1.7
	M1-2	2.6	2.0	2.2
	M1-6	3.2	1.5	−5.0
	M9-2	2.0	2.3	0.4
IS working solution at 4 °C, 28 d	IS	2.1	4.5	1.2

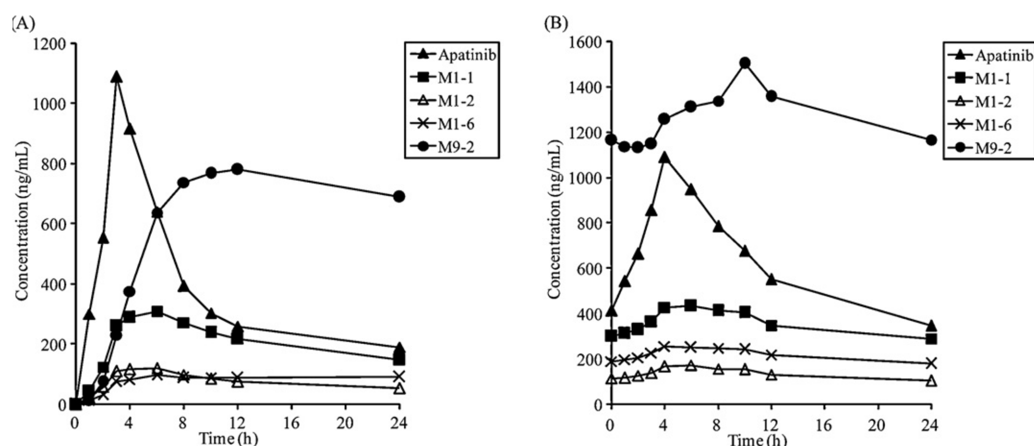

Fig. 3. Mean plasma concentration–time profiles of apatinib, M1-1, M1-2, M1-6, and M9-2 following oral administration of 500 mg apatinib mesylate once daily for 28 d in 5 patients with advanced colorectal cancer on day 1 (A) and day 28 (B).

Table 6
The main pharmacokinetic parameters of apatinib and its four major metabolites following oral administration of 500 mg apatinib mesylate once daily for 28 d in patients with advanced colorectal cancer.

Time	Parameter	Apatinib	M1-1	M1-2	M1-6	M9-2
Day 1	C_{max} (ng/mL)	1146 ± 303	322 ± 76.6	127 ± 35	109 ± 35	803 ± 385
	T_{max} (h)	3.0 ± 0.7	6.0 ± 1.4	4.6 ± 1.3	10.0 ± 8.1	13.6 ± 5.9
	AUC_{0-24h} (ng h/mL)	8991 ± 3139	4932 ± 1317	1822 ± 622	1976 ± 690	14,774 ± 6916
Day 28	C_{max} (ng/mL)	1234 ± 469	471 ± 150	186 ± 57	282 ± 66	1582 ± 783
	T_{max} (h)	3.3 ± 1.5	6.3 ± 3.9	4.3 ± 2.4	5.3 ± 3.4	6.0 ± 5.9
	AUC_{0-24h} (ng h/mL)	14,649 ± 6407	8473 ± 2296	3195 ± 947	5208 ± 801	30,672 ± 12,849

following oral administration of 500 mg apatinib mesylate once daily for 28 d. The mean plasma concentration–time curves of apatinib, M1-1, M1-2, M1-6, and M9-2 are presented in Fig. 3, and the pharmacokinetic parameters are listed in Table 6.

3.6. Incurred sample reanalysis

ISR passed for this study with 95%, 100%, 90%, 80%, and 100% of ISR samples, meeting the acceptance criterion for apatinib, M1-1, M1-2, M1-6, and M9-2, respectively.

4. Conclusion

A simple, sensitive, selective, and reproducible LC–MS/MS method was successfully developed and validated for the simultaneous determination of apatinib and its four major metabolites (M1-1, M1-2, M1-6, and M9-2) in human plasma. Gradient elution was selected to obtain good resolution and maintain the retention of all the analytes in a short chromatographic time. The linear range of this method was 3.00–2000 ng/mL for each analyte. The LLOQ of this method was 3.00 ng/mL for each analyte. A chromatographic total run time of 9 min was achieved. Sample preparation was simple and quick with acetonitrile protein precipitation. The method could be applied as a routine assay in evaluating the exposure of apatinib and its major metabolites in human pharmacokinetic studies.

References

[1] J. Glade-Bender, J.J. Kandel, D.J. Yamashiro, Expert Opin. Biol. Ther. 3 (2003) 263.
[2] S. Tian, H.T. Quan, C.Y. Xie, H.Y. Guo, F.F. Lü, Y.P. Xu, J. Li, L.G. Lou, Cancer Sci. 102 (2011) 1374.
[3] P. Chen, L. Iruela-Arispe, L.G. Lou, P.Y. Sun, K.H. Yuan, Proc. Am. Assoc. Cancer Res. 47 (2006), Abst. 1764.
[4] J. Li, X.M. Zhao, L. Chen, H.Y. Guo, F.F. Lv, K. Jia, K. Yv, F.Q. Wang, C. Li, J. Qian, C.L. Zheng, Y.X. Zuo, BMC Cancer 10 (2010) 529.
[5] FDA, Guidance for Industry: Bioanalytical Method Validation, 2001, http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM070107.pdf (last accessed May 2011).
[6] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 75 (2003) 3019.
[7] C.T. Viswanathan, S. Bansal, B. Booth, A.J. DeStefano, M.J. Rose, J. Sailstad, V.P. Shah, J.P. Skelly, P.G. Swann, R. Weiner, AAPS J. 9 (2007) E30.