A specific and sensitive liquid chromatography–electrospray ionization–tandem mass spectrometric method was developed for the quantification of imatinib and its primary metabolite N-desmethyl imatinib in human plasma. Protein precipitation with methanol was used for sample preparation. High-performance liquid chromatographic separation was performed on a Thermo BDS Hypersil C18 column (4.6 × 100 mm, 2.4 μm) with methanol–water (55:45, v/v) containing 0.1% formic acid and 0.2% ammonium acetate as the mobile phase, using isocratic elution at a flow rate of 0.7 mL/min. Detection was conducted with positive electrospray ionization multiple reaction monitoring of the ion transitions at m/z 494 → 394 for imatinib, 480 → 394 for N-desmethyl imatinib and 297 → 110 for the internal standard (palonosetron). The assay was validated in the concentration ranges of 8–5,000 ng/mL for imatinib and 3–700 ng/mL for N-desmethyl imatinib. The quantification limits for imatinib and N-desmethyl imatinib were 8 and 3 ng/mL, respectively. The intra-day and inter-day precision values of the assay (expressed as percentage relative standard deviation) were less than 15% at all concentration levels within the tested range, and the accuracy values were between 85 and 115%. The established method was successfully applied to the pharmacokinetic study of imatinib mesylate capsules in 24 healthy Chinese volunteers.
Methods

Instrumentation and conditions
The LC–MS–MS system consisted of a Waters 2695 HPLC system (Waters, Milford, MA) with a quaternary gradient pump, an online vacuum degasser, a column oven and an autosampler, coupled to a Micromass Quattro micro triple-quadrupole mass spectrometer (Micromass, Manchester, UK) equipped with an electrospray ionization (ESI) interface. Data acquisition was performed with Masslynx 4.0 software (Micromass).

HPLC separation was performed on a Thermo BDS Hypersil C18 column (4.6 × 100 mm, 2.4 μm) maintained at 40°C with a mobile phase of methanol–water containing 0.1% formic acid and 0.2% ammonium acetate (55:45, v/v), which was delivered at 0.7 mL/min; 30% of the eluent was split into the inlet of the mass spectrometer for detection. A divert valve was used to divert the eluent to waste from 0 to 2.4 min. The autosampler was set at 4°C.

The mass spectrometer was operated in the positive ESI mode with the spray voltage set at 3 kV, nitrogen gas desolvation flow of 500 L/h at a temperature of 350°C and a sweep gas flow of 20 L/h. Quantification was performed with multiple reaction monitoring (MRM) by using argon gas collision induced dissociation and the following ion transitions: m/z 494 → 394, 480 → 394 and 297 → 110 for imatinib, N-desmethyl imatinib and palonosetron (IS), respectively, with the cone voltages all set at 50 V and the collision energy at 28 eV. Figure 1 shows the typical production scan spectra and the proposed patterns of fragmentation of the analytes and the IS.

Stock solutions
Stock solutions of imatinib and N-desmethyl imatinib at concentrations of approximately 800,000 and 90,000 ng/mL, respectively (both as the free base), were prepared in methanol–water (1:1, v/v). Working solutions of imatinib and N-desmethyl imatinib were prepared by serial dilution with the same solvent in the range from 80 to 50,000 ng/mL for imatinib and 30 to 7,000 ng/mL for N-desmethyl imatinib. The stock and working solutions of palonosetron hydrochloride (IS) were prepared similarly at 200,000 and 2,000 ng/mL as the free base. All solutions were stored under refrigeration (4°C) when not in use.

Plasma sample pretreatment
An aliquot of a 0.4 mL plasma sample was spiked with 40 μL of the IS and 40 μL of methanol–water (1:1, v/v), or 40 μL of the corresponding working standard solutions, for the preparation of the calibration plasma standards and quality control samples, followed by protein precipitation with the addition of 1.2 mL methanol and vortex-mixing for 1 min and centrifuging at 10,000 × g for 10 min at 4°C. The supernatant was transferred into an autosampler vial for LC–MS–MS analysis with an injection volume of 10 μL.

The calibration plasma standards of imatinib and N-desmethyl imatinib were prepared and analyzed separately to avoid possible cross-talk interferences, although these were not found in this study. To prepare the plasma calibration standards, an aliquot of 40 μL of each working standard solution was mixed precisely with 0.4 mL of blank plasma to produce the calibration standard in the ranges of 8.0–5000 ng/mL for imatinib and 3.0–700 ng/mL for N-desmethyl imatinib. The quality control (QC) samples were made at 16, 400 and 3,200 ng/mL for imatinib and 9, 90 and 500 ng/mL for N-desmethyl imatinib in the same way.

Method validation
The analytical method was validated for specificity, matrix effects, linearity, lower limit of quantification (LLOQ), accuracy, precision and recovery of measurements. The specificity was evaluated by comparing the chromatograms of six different batches of blank plasma with the corresponding spiked plasma to investigate the potential interferences near the retention times of either the analytes or the IS. The linearity of the method was determined by the analysis of a series of standard samples with concentrations from 8.0 to 5,000 ng/mL for imatinib and 3.0 to 700 ng/mL for N-desmethyl imatinib. The calibration curves were established through weighted linear least-squares regression of the peak area ratios (Y) of the analytes to the IS obtained against the corresponding concentrations (C, in ng/mL). Coefficients of correlation (r) were required to be 0.99 or better. The acceptance criterion for each back-calculated standard concentration above the LLOQ was ±15% deviation from the nominal value, except at LLOQ. The LLOQ was defined as the concentration of the sample that could be quantified with less than 20% variation in precision (n = 6) and provided a signal-to-noise ratio ≥ 10; this was established by using six independent samples. The intra-batch and inter-batch accuracy and precision were determined by analysis of five replicates at three QC concentration levels. The criteria for acceptability of the data included accuracy within ±15% deviation from the nominal values and precision within 15%. Recoveries of imatinib and N-desmethyl imatinib from plasma with protein precipitation by methanol were determined by comparing their peak areas in spiked plasma samples at three QC concentrations with those in samples prepared by spiking the blank plasma post-preparation with the same amounts of imatinib and N-desmethyl imatinib. The recovery of the IS was evaluated at 2,000 ng/mL. Matrix effects were caused by ionization competition occurring among imatinib, N-desmethyl imatinib, IS and endogenous co-eluting components. To evaluate the matrix effects, chromatographic peaks of imatinib, N-desmethyl imatinib and IS from the spiked solution after preparation were compared with those obtained by direct injection of the standard solutions prepared in the mobile phase at the QC concentrations.

The stability of the analytes was assessed by using triplicate spiked plasma samples containing imatinib and N-desmethyl imatinib at two concentration levels (30 and 1,600 ng/mL for imatinib; 18 and 350 ng/mL for N-desmethyl imatinib), which were analyzed after subjection to various storage and handling conditions over time periods that exceeded those applied to the actual study samples. The spiked stability samples were analyzed against a calibration curve that was obtained from spiked calibration standards prepared from freshly made stock solutions; the obtained concentrations were compared to the nominal concentrations. The mean concentration at each level should be within ±15% of the nominal concentration. For freeze–thaw stability, samples were stored at −20°C for 24 h and thawed unassisted at room temperature. After complete thawing, samples were refrozen again under the same conditions. The freeze–thaw cycle was repeated three times and analysis was conducted.
on the third cycle. Short-term temperature stability was assessed by analyzing samples thawed at room temperature and kept at this temperature for 8 h, and the stability of the post-preparative samples kept at room temperature for 8 h was also evaluated. The stability of the post-preparative samples in the autosampler was conducted by re-analyzing processed samples kept in the autosampler at 4°C for 24 h. Long-term stability was determined by storing at −20°C for 75 days.

The stabilities of stock solutions of imatinib, N-desmethyl imatinib and IS (with an appropriate dilution, taking into consideration the linearity and measuring range of the detector) were evaluated by comparing the response of the stock solutions kept at 4°C for 50 days with that of freshly prepared solutions.

Method application

The validated method was applied for the determination of imatinib and its primary metabolite N-desmethyl imatinib in plasma samples in a pharmacokinetic study. Twenty-four healthy male Chinese volunteers were selected as subjects after clinical screening procedures. Each subject was fasted and administered a single oral dose of 400 mg of imatinib mesylate capsules. Venous blood samples of approximately 4 mL were collected into heparinized polypropylene tubes at pre-dose and 0.5, 1.0, 1.5, 2.5, 4.0, 6.0, 8.0, 12, 24, 36, 48, 72, 96 and 120 h after administration. Plasma was separated by centrifugation at 4,000 g for 5 min and stored at −20°C until analysis. The study was conducted at Xijing Hospital (Xi’an, China) in accordance with the
principles of the Declaration of Helsinki after receiving approval from the independent ethics committee in the hospital. All subjects gave written consent for their participation after having been informed by the medical supervisor about the aim, course and possible risks of the study.

Results

**Method validation**

Under the proposed LC–MS-MS conditions, retention times for imatinib, N-desmethyl imatinib and the IS were 2.8 ± 0.1, 2.9 ± 0.1 and 2.8 ± 0.1 min, respectively, and the total run time was
approximately 3.8 min. No obvious interferences from endogenous substances were observed. Typical chromatograms are shown in Figure 2 for blank plasma, plasma spiked with LLOQ standards of imatinib, N-desmethyl imatinib and IS, and plasma samples collected from a subject 4 h after dosing.

Suitable weighting factors were selected for linear regression because the F-tests and homoscedasticity tests, conducted by plotting residuals versus concentration, demonstrated the heteroscedasticity. Empirical weights of 1/Y, 1/Y², 1/Y³ and 1/C² were evaluated. The best weighting factor was chosen according to the percentage relative error (RE), which compares the regressed concentration computed from the regression equation obtained for each weighting factor with the nominal standard concentration. Results showed that the weighting factor of 1/C gave the least sum of absolute RE across the whole concentration range; thus, it was selected as the weighting factor. Good linear relationships were obtained over the ranges of 8–5000 and 3–700 ng/mL for imatinib and N-desmethyl imatinib, respectively. Typical equations were Y = (2.188 ± 0.006028) C + (0.1794 ± 0.01397) (r = 0.9997 ± 0.0001) (n = 3) for imatinib and Y = (1.258 ± 0.01908) C + (0.01479 ± 0.001946) (r = 0.9997 ± 0.0001) (n = 3) for N-desmethyl imatinib. The accuracy observed for the mean of back-calculated concentrations for three calibration curves was within 97.26–106.3% and 90.90–106.2% for imatinib and N-desmethyl imatinib, respectively, whereas the inter-validation precision [percentage relative standard deviation (RSD)] of the back-calculated calibration standards ranged from 0.28 to 2.15% for imatinib and 0.10 to 3.28% for N-desmethyl imatinib. The accuracy, precision (RSD) and regression parameters of slope, intercept and correlation coefficient (r) calculated by weight (1/C) linear regression are summarized in Tables I and II. The LLOQ values were found to be 8 and 3 ng/mL for imatinib and N-desmethyl imatinib, respectively; these values are in agreement with the requirement in human pharmacokinetic study.

The intra-batch and inter-batch precision values were ≤8.3% for imatinib and ≤5.1% for N-desmethyl imatinib. The accuracy, expressed as deviation percentage, was found to be within the acceptable range. The recovery data show that the sample preparation method was able to produce consistent, precise, reproducible and absolute recovery for the analytes and IS (>97%). No obvious matrix effects were found for the analytes and IS: the ratios of the peak responses ranged from 85 to 115%, which were within the acceptable limits. The results of accuracy, precision, recovery and matrix effect of imatinib and N-desmethyl imatinib are summarized in Table III.

The stock solutions of imatinib, N-desmethyl imatinib and IS were stable under the specified conditions for 50 days with a percentage bias of less than 2.5%. No significant changes (< 8.0%) in the concentrations of imatinib or N-desmethyl imatinib were observed after three freeze-thaw cycles. After storage at room temperature for 8 h, the concentrations of the analytes in plasma deviated less than ±4.4% from their nominal concentrations. No significant loss (<5.0%) of either analyte was observed after storage at −20 C for 75 days. The post-preparative plasma samples were stable at room temperature for 8 h, as shown because the concentrations varied no more than ±5.9% at their nominal concentrations. When kept at 4 C in the autosampler, post-preparative plasma samples showed good stability, with concentrations that varied no more than ±5.4% after 24 h of storage.
The metabolite, \( N \)-desmethyl imatinib, could be detected at all time points over the duration of 0.5–120 h. The area under the curve from zero to infinity (AUC\(_{0\rightarrow\infty}\)) value for \( N \)-desmethyl imatinib (5,848 \( \pm \) 1,521 ng h/mL) was only approximately 14.0% that of imatinib (3,8091 \( \pm \) 7,607 ng h/mL), indicating that only a small amount of imatinib was metabolized to \( N \)-desmethyl imatinib following oral administration of imatinib mesylate capsules. The time after administration of the drug when the maximum plasma concentration was reached (\( T_{\text{max}} \)) for \( N \)-desmethyl imatinib was the same as that of imatinib, approximately 2.8 h; therefore, the metabolism was initiated along with the absorption. The pharmacokinetic parameters found in this study for the Chinese subjects were in good agreement with those found in other populations (21–22). Therefore, there are no obvious racial differences in the pharmacokinetics of imatinib mesylate.

### Discussion

**LC–MS–MS analysis**

A reverse HPLC system was chosen for the bioassay according to the chemical and chromatographic features of the analytes. HPLC separation was established on a Thermo BDS Hypersil C18 column \((4.6 \times 100 \text{ mm}, 2.4 \mu \text{m})\), which attained good resolution, high capacity and high throughput goals. The compositions of the mobile phase were optimized to achieve the best peak shape and mass spectrometric responses for the analytes and the IS in a minimum run time. It was found that the addition of formic acid provided better symmetrical peak shapes for imatinib and \( N \)-desmethyl imatinib, and the addition of ammonium acetate enhanced the retention of the analytes, whereas a higher ratio of methanol content in the mobile phase was also necessary for sensitive positive ESI-MS detection. As a result, an isocratic elution was selected using a mobile phase of methanol–water containing 0.1% formic acid and 0.2% ammonium acetate (55:45, v/v) at a flow rate of 0.7 mL/min with a total run time of only 3.8 min; this resulted in steady chromatographic retention, improved instrumental responses, elimination of matrix effects and suitability for high-throughput bioanalysis.

Mass spectrometric conditions were optimized to achieve maximum stable responses of the parent and major product ions of the analytes. Continuous flow analysis was conducted to obtain parent and product ion mass spectra of imatinib, \( N \)-desmethyl imatinib and IS. The analytes and IS gave predominant singly charged protonated precursor [\( M + H \)^+] ions at \( m/z \) of 494, 480 and 297 for imatinib, \( N \)-desmethyl imatinib and IS, respectively, in Q1 full scan spectra. Following optimization of the mass spectrometry conditions, the most abundant product ions with \( m/z \) of 394, 394 and 110 were chosen for MRM detection of imatinib, \( N \)-desmethyl imatinib and IS, respectively.

### Conclusion

A specific and sensitive LC–MS–MS method was developed and fully validated for the simultaneous determination of imatinib and its active metabolite \( N \)-desmethyl imatinib in human plasma. The major advantages of this validated method included the simple and rapid sample treatment, the excellent recovery, the minor matrix effect and the runtime of 3.8 min, which allowed for high throughput analysis of numerous samples. The method was successfully applied to a pharmacokinetic study in 24 healthy Chinese volunteers, and the primary pharmacokinetic parameters of imatinib and \( N \)-desmethyl imatinib in the Chinese population were reported for the first time.

### Method application

The method was successfully applied to determine imatinib and its primary metabolite \( N \)-desmethyl imatinib in human plasma during a pharmacokinetic study in Chinese subjects, the biometric data for which are given in Table IV. Generally, the subjects of pharmacokinetic studies should belong to either sex to indicate a possible sex influence. The absence of sex differences in the pharmacokinetics of imatinib has been confirmed in published studies (3, 20); therefore, the present study was conducted on male subjects. The strategy of blood sampling was in accordance with good clinical practices and was carefully explained to patients and investigators. Approximately 4 mL of blood was collected at each sampling point; the total volume of blood drawn from each subject completing this study did not exceed 2% of circulating blood volume, which was not likely to cause impairment to the healthy volunteers or the study results. The pharmacokinetic parameters were estimated by using the non-compartmental method. The mean plasma concentration–time curves of imatinib and its metabolite \( N \)-desmethyl imatinib are presented in Figure 3. The estimated pharmacokinetic parameters are shown in Table V. The metabolite, \( N \)-desmethyl imatinib, could be detected at all time points over the duration of 0.5–120 h. The area under the curve from zero to infinity (AUC\(_{0\rightarrow\infty}\)) value for \( N \)-desmethyl imatinib (5,848 \( \pm \) 1,521 ng h/mL) was only approximately 14.0% that of imatinib (3,8091 \( \pm \) 7,607 ng h/mL), indicating that only

**Figure 3.** The mean plasma concentration–time curves of imatinib (squares) and its metabolite \( N \)-desmethyl imatinib (circles) after a single oral dose of imatinib mesylate capsules (400 mg of imatinib free base) (mean \( \pm \) SD, \( n = 24 \)).

**Table V**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Imatinib</th>
<th>( N )-desmethyl imatinib</th>
</tr>
</thead>
<tbody>
<tr>
<td>C(_{\text{max}}) (ng/mL)</td>
<td>1,929 ( \pm ) 479</td>
<td>194 ( \pm ) 59</td>
</tr>
<tr>
<td>( T_{\text{max}}) (h)</td>
<td>2.8 ( \pm ) 1.2</td>
<td>2.8 ( \pm ) 1.2</td>
</tr>
<tr>
<td>T(_{1/2}) (h)</td>
<td>17.6 ( \pm ) 1.6</td>
<td>37.6 ( \pm ) 3.5</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>23.5 ( \pm ) 2.3</td>
<td>48.0 ( \pm ) 3.7</td>
</tr>
<tr>
<td>AUC(_{0\rightarrow\infty}) (ng h/mL)</td>
<td>37,775 ( \pm ) 7,575</td>
<td>5,275 ( \pm ) 1,380</td>
</tr>
<tr>
<td>AUC(_{0\rightarrow\infty}) (ng h/mL)</td>
<td>38,081 ( \pm ) 7,607</td>
<td>5,848 ( \pm ) 1,521</td>
</tr>
<tr>
<td>CL/F (L/h)</td>
<td>123 ( \pm ) 3.7</td>
<td>82.9 ( \pm ) 29.3</td>
</tr>
<tr>
<td>Vd/F (L)</td>
<td>299.1 ( \pm ) 47.3</td>
<td>4,713.9 ( \pm ) 2,157.3</td>
</tr>
<tr>
<td>(AUC(<em>{0\rightarrow\infty}) - desmethyl imatinib) / (AUC(</em>{0\rightarrow\infty}) imatinib) (%)</td>
<td>14.0 ( \pm ) 2.3</td>
<td>—</td>
</tr>
</tbody>
</table>

Note: C\(_{\text{max}}\) = maximum plasma concentration; \( T_{1/2}\) = half-life; MRT = mean residence time; CL/F = clearance; Vd/F = volume of distribution.