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Development and validation of a liquid chromatography/tandem mass spectrometry procedure for the quantification of sunitinib (SU11248) and its active metabolite, *N*-desethyl sunitinib (SU12662), in human plasma: Application to an explorative study

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ABSTRACT

A sensitive, precise and accurate quantitative liquid chromatography/tandem mass spectrometry (LC–MS/MS) method for the measurement of sunitinib (SU11248) and *N*-desethyl sunitinib (SU12662) in human plasma was developed and validated. All sample handling was done under strict light protection. The sample preparation method employed acetonitrile protein precipitation using d_5-SU11248 as an internal standard. The processed samples were chromatographed on a polymeric reversed-phase analytical column and analyzed by triple-quadrupole MS/MS in multiple reaction monitoring (MRM) mode using positive TurboIonSpray® (TISP). The LC–MS/MS method described in this paper presents high absolute recovery (86.2% SU11248, 84.8% SU12662), high sensitivity (lower limit of quantitation of 0.06 ng/mL for both analytes), high inter-day precision (1.6–6.1% SU11248, 1.1–5.3% SU12662) and high analytical recovery (99.8–109.1% SU11248, 99.9–106.2% SU12662), as well as excellent linearity over the concentration range 0.060–100 ng/mL (r^2 > 0.999) with a short runtime of only 4.0 min. Results on the stability of SU11248 and SU12662 in human plasma are presented. During validation plasma from intensive care patients receiving many drugs were tested for interference and incurred samples were analyzed. The method met all criteria of the EMA and FDA guidelines during validation and was successfully applied to a pharmacokinetic study in healthy human volunteers.

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1. Introduction

Sunitinib (SU11248) is an oral small-molecule inhibitor of multiple tyrosine kinases with antitumor and antiangiogenetic effects, which is approved for the treatment of gastrointestinal stromal tumor (GIST) after disease progression or intolerance of imatinib therapy and for advanced renal cell carcinoma (RCC). The activity of sunitinib is based on the inhibition of vascular endothelial growth factor receptors 1–3 (VEGFR1-3), platelet-derived growth factor receptors (PDGFRα and PDGFRβ), steam cell factor receptor (KIT), fsm related tyrosine kinase-3 (FLT3), and colony-stimulating factor-1 receptor (CSF-1R) [1–5]. Targeting protein kinases has a greater specificity and fewer side effects than the traditional cytotoxic therapy (‘magic bullet’ concept of Paul Ehrlich) [6].

Sunitinib has a pK_a-value of 8.95 and is metabolized by the P450 enzyme CYP3A4 to *N*-desethyl sunitinib (SU12662), which is equipotent to the parent compound and has an exposure that is between 23% and 37% of the total exposure. Protein binding of sunitinib and *N*-desethyl sunitinib is 95% and 90%, respectively. AUC and C_{max} are increasing proportionally with increasing dose within a range of 25–100 mg and are not affected by food. Maximum plasma concentrations of sunitinib are reached between 6 and 12 h and the terminal half-lives of sunitinib and *N*-desethyl sunitinib are 40–60 h and 80–110 h, respectively [7–10].

Table 1 shows analytical methods for the quantification for sunitinib and *N*-desethyl sunitinib in human plasma and monkey tissues [10–15]. Liquid chromatography coupled to MS, MS/MS or UV are the methods of choice depending on the required sensitivity, the biological matrix, and the applied extraction/workup procedures, *e.g.* liquid–liquid or solid phase extraction, or protein precipitation. One issue always encountered when analyzing samples containing either sunitinib or *N*-desethyl sunitinib is their susceptibility

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Table 1
Validated analytical methods for the determination of sunitinib (SU11248) and *N*-desethyl sunitinib (SU12662).

Method	Linearity range [ng/mL]	Approx. runtime [min]	Sample volume [mL]	Sample preparation	Inter-day CV [%]	Inter-day relative error [%]	Recovery [%]	Correlation coefficient	Internal standard	Determination of *N*-desethyl sunitinib	Additional validation experiments[a]
Present method LC–MS/MS	0.06–100	4.0	0.1	PP	1.6–6.1 (SU11248) 1.1–5.3 (SU12662)	0.2–9.1 (SU11248) −0.1–6.2 (SU12662)	86.2 ± 6.4 (SU11248) 84.8 ± 5.0 (SU12662) 85.3 ± 0.8 (IS)	>0.999 (SU11248) >0.999 (SU12662)	d_5-SU11248	+	1; 2a-d; 3; 4a,b; 5; 6; 7; 9
de Bruijn et al. [29] LC–MS/MS	0.200–50.0	4.0	0.1	LLEx	1.14–6.52 (SU11248) 1.15–5.95 (SU12662)	−9.5–0.7 (SU11248) −6.0–106.8 (SU12662)	101 ± 7.9 (SU11248) 102 ± 7.9 (SU12662) 105 ± 6.7 (IS)	≥ 0.9980	d_{10}-SU11248	+	1; 2a,c,d,f; 6; 9; 10
Zhou et al. [17] LC–MS/MS	1.37–1000	3.2	0.01	PP	1.7–4.9 (SU11248)	4.2–12.7 (SU11248)	93.1–96.1 (SU11248) 93 (IS)	≥0.99	Camptothecin	–	1; 2d
Honeywell et al. [26] LC–MS/MS	1–4000	<4.0	0.02	PP	4.3–6.0 (SU11248)	0.2–11.3 (SU11248)	74.3–78.7 (SU11248)	>0.99 (SU11248)	n.r.	–	1; 2b
Bello et al. [10] LC–MS	0.1–n.r.	n.r.	n.r.	LLEx	2.4–6.5 (SU11248) 3.7–11.1 (SU12662)	−1.3–1.3 (SU11248) −1.7–2.3 (SU12662)	n.r.	n.r.	d_{10}-SU11248	+	n.r.
Barattè et al. [11] LC–MS/MS	2–2000 ng/g	5.0	50 mg	LLEx	2.4–9.6 (SU11248) 4.5–7.6 (SU12662)	−1.3–2.2 (SU11248) −9.4–2.1 (SU12662)	n.r.	>0.990 (SU11248) >0.988 (SU12662)	d_{10}-SU11248	+	1; 2a,c,e; 3
Haouala et al. [12] LC–MS/MS	1–500	14.0	0.1	PP	1.3–6.1 (SU11248)	−5.4 – (−0.3 (SU11248)	91.3–96.8	>0.99 (SU11248)	d_{10}-SU11248	–	1; 2a,b,d; 6; 8
Minkin et al. [13] LC–MS/MS	0.2 –500	3.0	0.2	LLEx	2.2–10.1 (SU11248)	−3.3–5.8 (SU11248)	39.2–46.1 (SU11248) 63 (IS)	>0.995	Clozapine	–	1; 2a–e
Blanchet et al. [14] LC–UV	20 –200	10.0	1.0	LLEx	<7	0.2–5.9	50.0–70.5 18.2 (IS)	>0.995	Ranitidine	–	1; 2a–e; 9
Etienne-Grimaldi et al. [15] LC–UV	5.0 (SU11248)/ 2.5 (SU12662) –250	14.0	0.5	LLEx	6.4 –14.5 (SU11248) 8.3–10.0 (SU12662)	n.r.	59.0–63.2 (SU11248) 80.4–92.8 (SU12662)	>0.993 (SU11248) >0.998 (SU12662)	Vandetanib	+	1; 2d,f

n.r.: not reported, PP: protein precipitation, LLEx: liquid–liquid extraction.

[a] 1: specificity; 2: stability (short-term (a), long-term (b), post-preparative (c), freeze-thaw (d), stock solution (e), light (f)); 3: influence of dilution; 4: influence of hemolyzed (a), lipemic (b) plasma; 5: influence of different individuals; 6: matrix effect; 7: incurred samples; 8: influence of anticoagulants; 9: influence of drugs in patient matrix; 10: interference of drugs in human EDTA plasma.

towards light-induced *E*/*Z*-isomerism. Both, sunitinib and *N*-desethyl sunitinib belong to a class of tyrosine kinase inhibitors that can be referred to as 3-((pyrrol-2-yl)methylidenyl)indolin-2-ones, *i.e.* sunitinib and *N*-desethyl sunitinib are 5-fluoro-2-oxindoles attached to a dimethyl pyrrole carboxamide by an exocyclic double bond prone to showing *E*/*Z*-isomerism. Sun et al. reported that such compounds are usually obtained solely as *Z*-isomers, whereas analogous 3-benzylidenylindolin-2-ones show *E*/*Z*-isomerism [16]. The predominance of *Z*-isomerism for the 3-((pyrrol-2-yl)methylidenyl)indolin-2-ones results from an intramolecular hydrogen bond between the C-2 carbonyl oxygen atom of the indolin-2-one ring and the proton of the pyrrole NH-group. However, large-scale production and drug formulation may lead to the *E*-isomer. Both isomers were detected in pharmaceutical preparations at ratios of about 1:2, as well as in patients' plasma samples at variable ratios [12]. Equilibrium formation between the *Z*- and *E*-isomers was observed in polar solvents and as a result of pH, as well as by exposure to light [16–18]. Therefore special attention must be paid to both isomers during analysis. If significant amounts of the *E*-isomer are observed, they must be considered, *e.g.* by summation of the peak areas of both isomers [12], and exposure to light must be kept at a minimum during sample preparation. However and in contrast to isomerization prior to chromatography, isomerization during chromatography has not been observed by LC–MS/MS analysis of either sunitinib, d_5-sunitinib or *N*-desethyl sunitinib [19]. As a result of strict protection from light during sample preparation and by use of neat *Z*-isomer reference substances, no significant *E*/*Z*-isomerism was observed in the present method.

In this paper, we report a highly sensitive LC–MS/MS method for the determination of sunitinib and *N*-desethyl sunitinib with an LLOQ of 0.060 ng/mL. To our knowledge this is the most sensitive LC–MS/MS method for the determination of sunitinib and *N*-desethyl sunitinib with a runtime of only 4 min. The assay was fully validated according to GLP, FDA [20] and EMA [21] guidelines.

2. Experimental

2.1. Chemicals and reagents

Sunitinib was purchased from LC Laboratories (Woburn, MA, USA). *N*-desethyl sunitinib and d_5-sunitinib were synthesized according to literature procedures [19]. In brief, the synthesis of *N*-desethyl sunitinib and d_5-sunitinib started from 5-fluoroindolin-2-one, which was readily obtained by a Wolff–Kishner-type reduction of 5-fluoroisatin. Ethyl 2,4-dimethyl-1*H*-pyrrole-3-carboxylate (Ace Synthesis, Woburn, USA) was formylated under Vilsmeier conditions and, after alkaline hydrolysis of the ethyl ester moiety, condensed with 5-fluoroindolin-2-one in a pyrrolidine-catalyzed aldol reaction. *N*-desethyl sunitinib was finally obtained through an EDC/HOBT coupling reaction with *N*-ethylethylenediamine at −60 °C. For d_5-sunitinib, *N*-ethylethylenediamine was phthalimide-protected in a Gabriel type of reaction and subsequently alkylated with $[^2H_5]$ethyl iodide. Hydrazinolysis provided *N*,*N*-$[^2H_5]$diethylethylenediamine, which was reacted again in an EDC/HOBT coupling reaction to afford d_5-sunitinib. Acetonitrile was of HPLC-grade and from FSA Laboratory Supplies (Loughborough, United Kingdom). Other chemicals used were of analytical grade and from VWR International GmbH (Darmstadt, Germany). Ultra pure water was obtained using a Milli-Q purification system (Millipore Corporation, Bedford, MA, USA).

2.2. Non-hemolyzed and hemolyzed plasma samples

Drug-free human plasma using EDTA as the anticoagulant was used for method validation. Hemolyzed plasma was prepared by adding 1% of frozen and thawed drug-free blood to drug-free human plasma using EDTA as the anticoagulant.

2.3. Calibration standards and spiked quality control standards

For validation of the sunitinib and *N*-desethyl sunitinib method a human plasma calibration curve of 11 standards, including a blank sample, which was not used for calculation of linear regression, and spiked quality control standards (SQC 1 to SQC 5) were prepared each validation day. During pharmacokinetic studies it occurs very frequently that concentrations above the upper limit of quantitation occur. Therefore, on one validation day, a sample with a high concentration of sunitinib and *N*-desethyl sunitinib, significantly above the upper limit of quantitation, was prepared separately to test the effect of dilution on response and accuracy. That sample was diluted fivefold with blank human plasma prior to workup to control the precision and accuracy of the assay for samples with concentrations above the upper quantification limit that should be diluted prior to analysis. For calibration during measurement of study samples, a calibration curve of 11 standards (including a blank sample, which was not used for calculation of linear regression) and spiked quality control standards (SQC 1 to SQC 4) were prepared to control the precision and accuracy of the assay during the measurement of study samples.

On each validation day two stock solutions (I, II) for sunitinib and two stock solutions (I, II) for *N*-desethyl sunitinib were prepared, each containing 100 μg/mL. Stock solutions (I) were used to prepare the calibration standards and stock solutions (II) were used to prepare the SQC samples. Calibration standards and spiked quality control samples were prepared freshly by serial dilution, adding the appropriate amount of stock solution of sunitinib and *N*-desethyl sunitinib (I or II) or the higher concentrated calibration standard and spiked quality control standard to drug-free human plasma, respectively.

2.4. Sample preparation

All sample handling was done under strict light protection and thawing of frozen plasma samples was performed in a water bath. Human plasma samples (100 μL) were deproteinized by addition of 200 μL of acetonitrile containing the internal standard. After thorough mixing, the samples were centrifuged at 3600 rpm (3016 × *g*) for 5 min at approximately +4 °C. Following centrifugation, 20 μL of each sample supernatant were injected onto the LC–MS/MS system.

2.5. Liquid chromatography/mass spectrometry

The liquid chromatography system consisted of a binary HPLC-Pump 1200 Series from Agilent Technologies (Waldbronn, Germany). Chromatographic separations were performed on a PLRP-S, 5 μm (50 × 4.6 mm I.D.) column (Agilent Technologies, Waldbronn, Germany). A PLRP-S column was chosen as it contains macroporous spherical polystyrene/divinylbenzene particles that are chemically and physically inert across the complete pH range, and, because of their minimal swell, may be used with all common organic modifiers in both isocratic and gradient elution mode. The mobile phase, delivered at a flow rate of 1.0 mL/min at ambient temperature, consisted of 50 mM ammonium formate buffer and acetonitrile (57:43, v:v) and 20 μL of sample were injected onto the LC–MS/MS system.

Detection was performed using an AB SCIEX API 5000™ triple quadrupole mass spectrometer (AB SCIEX, Concord, Ontario, Canada; in Germany supplied by: AB SCIEX Germany GmbH, Darmstadt) with TurboIonSpray® interface. High purity nitrogen gas was used as nebulizer, auxiliary, curtain and collision gas. The mass spectrometer was operated in positve ion mode using the following

settings: IS-Voltage: 2000 V, source temperature: 350 °C, declustering potential: 70 V (sunitinib, d_5-sunitinib), 40 V (*N*-desethyl sunitinib), collision energy: 33 eV (sunitinib, d_5-sunitinib), 26 eV (*N*-desethyl sunitinib), collision cell exit potential: 20 V. Gas settings: curtain gas 40 psi, GS1 50 psi, GS2 70 psi, collision gas thickness was set to 5.

Quantification was performed using multiple reaction monitoring (MRM) of the transitions m/z 399 → m/z 283 and m/z 371 → m/z 283 for sunitinib and *N*-desethyl sunitinib, respectively. For the internal standard the transition m/z 404 → m/z 283 was monitored. The dwell time per MRM transition was 400 ms for sunitinib, *N*-desethyl sunitinib and the internal standard with a pause time of 5 ms.

2.6. Data acquisition and processing

Data acquisition and processing was performed with Analyst Version 1.4.2 (AB SCIEX, Concord, Ontario, Canada). All software products were supplied by AB SCIEX Germany GmbH (Darmstadt, Germany). Calculations were performed with Microsoft Excel 2000 from Microsoft Co. (Redmond, WA, USA, 1985–2000).

2.7. Validation procedure

The method was validated according to the most recent European Medicines Agency (EMA) [21], U.S. Food and Drug Administration (FDA) guidelines [20], and our internal standard operation procedures. Specificity, linearity, lower limit of quantification (LLOQ), inter-day and intra-day precision and accuracy as well as absolute recovery and stability of sunitinib and *N*-desethyl sunitinib were evaluated. Possible interference on the determination of sunitinib and *N*-desethyl sunitinib by dilution or hemolyzed or lipemic plasma was studied, as well as the influence of different batches of human plasma and serum from healthy volunteers and severe ill patients [22–24]. Moreover, we examined the matrix effect on the determination and performed incurred sample analysis, the latter being a new requirement used in our laboratory before that has just been introduced into EMA guidance [21]. It requires the reanalysis of samples that have been analyzed before during a first run of clinical samples as 'the use of calibration standards and QC samples during validation may not mimic the actual study samples'. However, the number of samples that has to be reanalyzed is still a matter of debate. EMA guidance suggests that enough samples must be analyzed to assure 'at least sufficient confidence that the concentration being reported is accurate' (for details see Section 6 of the EMA guideline [21]). We reanalyzed 42 out of a total number of 379 samples, *i.e.* 11%.

2.7.1. Determination of specificity

The specificity of the sunitinib and *N*-desethyl sunitinib method was determined by screening twelve (six males and six females) different batches of control drug-free human plasma. The samples were prepared as previously described with and without addition of the internal standard. There should be no co-eluting peaks with areas of more than 20% of the analyte peak area at the LLOQ.

2.7.2. Evaluation of linearity, sensitivity, inter-day and intra-day precision and accuracy and lower limit of quantitation

For the determination of linearity, sensitivity, inter-day precision, quantification limit, and accuracy of the sunitinib and *N*-desethyl sunitinib assay a calibration curve and five sets of spiked quality control standards were prepared on each of the five validation days. The calibration curves were evaluated individually by linear regression and the concentrations of the calibration standards were back-calculated. The slopes, intercepts and the correlation coefficients of the corresponding individual curves were calculated. The calibration curves were accepted if there were not more than two outliers. If there were two outliers they had not to be adjacent. A calibration standard was defined as an outlier if the back-calculated concentration deviated more than 15% from the theoretical concentration at all concentrations except for the lowest concentration, where a deviation of less than 20% was acceptable. Means, standard deviations, coefficients of variation (%) and accuracy (%) were calculated for the back-calculated concentrations of each calibration standard.

The SQC samples were calculated by the corresponding calibration curve. The LLOQ, defined as the lowest calibration standard concentration at which both inter-day precision and accuracy were less than or equal to 20%, was evaluated. For the evaluation of the intra-day precision and accuracy of the assay, five sets of SQC samples of sunitinib and *N*-desethyl sunitinib in human plasma were analyzed on a validation day. The concentrations were calculated by the corresponding calibration curve. Means, standard deviations, coefficients of variation (%) and accuracy (%) were calculated for each SQC. Accuracy and precision were determined as relative error (RE, %) and coefficient of variation (CV, %) as follows: CV (%) = (standard deviation/mean) × 100, and RE (%) = (mean assayed conc. − theoretical conc.) × 100/theoretical conc. The inter- and intra-day CVs, as well as the accuracies for the spiked quality control samples should be within 15% except at the LLOQ, where a value of 20% is accepted.

2.7.3. Determination of absolute recovery of sunitinib, N-desethyl sunitinib and the internal standard in human plasma

For the determination of recovery of sunitinib, *N*-desethyl sunitinib and the internal standard, five spiked quality control standards in human plasma (SQC 1 to SQC 5, protein precipitation after spiking) and five spiked quality controls in processed blank human plasma (QBL 1 to QBL 5, protein precipitation before spiking) were prepared. Each SQC and each QBL was measured five times. The recovery of the analytes and the internal standards was evaluated according to the equation: recovery (%) = (mean peak area of analyte in spiked and processed human plasma samples/mean peak area of analyte added to processed blank human plasma) × 100.

2.7.4. Evaluation of stability

Stability of sunitinib and *N*-desethyl sunitinib in human plasma was assessed by analyzing SQC 1 and SQC 4, exposed to different conditions of time and temperature. The results were compared with those for freshly prepared SQC 1 and SQC 4 samples. The short-term stability was evaluated after exposure of the plasma samples to room temperature for 2 h and 4 h. The long-term stability was assessed after storage of the test samples at −20 °C and −70 °C. For each group, five replicates of each SQC sample were prepared and measured at day 0 and 5 months after the start of the stability test. The freeze/thaw stability was determined after three freeze/thaw cycles (−70 °C to room temperature) on consecutive days. The samples were frozen at −70 °C for 24 h and thawed at room temperature. After complete thawing the samples were refrozen at −70 °C for 12–24 h at −70 °C. This step was repeated two times. After the third thawing the samples were analyzed. The post-preparative storage stability of sunitinib and *N*-desethyl sunitinib was assessed at approximately +4 °C (autosampler temperature) for 24 h and 48 h after preparation.

Statistical evaluation was performed by calculating 95% ANOVA based confidence intervals for the ratios between the concentrations measured after given periods of time or after repeated thawing/freezing and the respective control (to allow for any contribution of assay imprecision). Instability was concluded if both the upper and lower limit of the confidence interval decreased by more than 10%.

2.7.5. Influence of dilution

The influence of a 1:5 dilution on the determination of sunitinib and *N*-desethyl sunitinib in human plasma was determined by measuring five samples of spiked quality control standard with high analyte concentrations in human plasma, which was at first diluted 1:5 with drug-free human plasma and than processed as previously described. The CV should be less or equal to 15%, the mean value within ±15% of the nominal value.

2.7.6. Influence of hemolyzed plasma

On a validation day the influence of hemolyzed plasma on the determination of sunitinib and *N*-desethyl sunitinib was determined by measuring five samples of spiked quality control standards number 1 and 4 in hemolyzed human plasma. The hemolyzed human plasma was processed as previously described for unhemolyzed human plasma. The spiked quality control standards in hemolyzed human plasma were analyzed together in the same run with the spiked quality control standards prepared in unhemolyzed human plasma. The CVs should be less or equal to 15%, the mean values within ±15% of the nominal values.

2.7.7. Influence of lipemic plasma

The influence of lipemic plasma on the determination of sunitinib and *N*-desethyl sunitinib was determined by measuring five samples of spiked quality control standard number 1 and 4 in lipemic human plasma, which were processed as previously described. The plasma used was collected after a high-fat breakfast desisting from an absolute determination of lipemia level. The spiked quality control standard in lipemic human plasma was analyzed together with the human unlipemic plasma spiked quality control standards in the same run. The CVs should be less or equal to 15%, the mean values within ±15% of the nominal values.

2.7.8. Influence of different batches of human plasma from healthy volunteers

On a validation day, the influence of six different batches of human plasma from healthy volunteers on the determination of sunitinib and *N*-desethyl sunitinib was investigated by measuring three samples of each spiked quality control standard number 1 and 4 in six different batches of human plasma, which were processed as previously described. The CVs should be less or equal to 15%, the mean values within ±15% of the nominal values.

2.7.9. Influence of different batches of human serum from severely ill patients

To determine the influence of human serum from severely ill patients (including neonates) on the determination of sunitinib and *N*-desethyl sunitinib, 25 human serum samples from severe ill patients who never received sunitinib were spiked with 50 ng/mL sunitinib and *N*-desethyl sunitinib and analyzed in triplicate. The CVs should be less or equal to 15%, the mean values within ±15% of the nominal values. Additionally, unspiked samples from the patients were analyzed to determine the specificity of the method regarding to serum samples containing other drugs than sunitinib and *N*-desethyl sunitinib. There should be no co-eluting peaks with areas of more than 20% of the analyte peak area at the LLOQ.

2.7.10. Precision in incurred human plasma samples

For the determination of the precision of sunitinib and *N*-desethyl sunitinib in incurred human plasma samples, *i.e.* samples from a pharmacokinetic study that have already been analyzed, we used the samples of a clinical study [25]. A total of 42 samples was analyzed a second time and compared to the results of the first analysis. The absolute differences in percent of the first to second analysis were calculated and the mean of these absolute differences in percent was calculated. The mean percentaged deviation should be within 20% of the absolute percentaged difference of the first and second analysis.

2.7.11. Matrix effect

The matrix effect was investigated with quantitative determination of the matrix factor (MF) and with post column infusion. To determine the MF for sunitinib and *N*-desethyl sunitinib the analytes and d_5-sunitinib were added to the mobile phase and six different drug free processed blank human plasma samples. Each sample was measured in triplicate. The area ratios of sunitinib/d_5-sunitinib and *N*-desethyl sunitinib/d_5-sunitinib were calculated in processed blank human plasma and the mobile phase. The MF was calculated by the following equation: MF = mean area ratio of SU11248 or SU12662 in processed blank human plasma/mean ratio of SU11248 or SU12662 in the mobile phase. The variability of the matrix factor, as measured by the CV, should be less than 15%. A matrix factor greater or smaller than one suggests analyte ion enhancement or suppression due to matrix components. A value of one signifies no matrix effect.

For determination of the matrix effect with post column infusion, a low concentration of sunitinib and *N*-desethyl sunitinib in the mobile phase was infused post column, and different batches of prepared blank human plasma were injected onto the chromatographic system. A decrease or increase of the signal reflects a suppression or enhancement of the analytes' ionization process.

2.7.12. Signal to noise

On the first 5 validation days, the signal to noise ratio is determined for sunitinib and *N*-desethyl sunitinib at the LLOQ (0.060 ng/mL). The mean value should be greater or equal to 5.

2.7.13. Use of the method during a pharmacokinetic study in human healthy volunteers

The LC–MS/MS procedure developed and validated here was used to generate the pharmacokinetic parameters of sunitinib and its active metabolite after administration of 50 mg sunitinib (Sutent™) once daily over 3 days (volunteers 1–4) and 5 days (volunteers 5–12) and have recently been reported [25]. In brief, the analytical procedure included blood collection before administration and after 1, 2, 4, 6, 8, 10, 12, 24, 25, 36, 48, 49, 60, 72, 96, 120, 240, 336 and 384 h after the first dose for volunteers 1-4 and additionally after 0.5, 24.5, 48.5, 72.5, 73, 96.5, 97, 98, 100, 102, 104, 106, 108, 144, 168 and 432 h after the first dose for volunteers 5-12. Samples of subjects for measurement of sunitinib and *N*-desethyl sunitinib were analyzed in a total of five sequences together with 10 calibration standards and 12 spiked quality control standards. Calibration standards (low to high concentrations) and quality control standards were measured within 9 h. The determined concentration of spiked quality control standards was compared to the theoretical concentration for accuracy.

3. Results and discussion

3.1. Full scan and product ion spectra of sunitinib, N-desethyl sunitinib and d_5-sunitinib

Solutions of sunitinib and *N*-desethyl sunitinib (10.0 ng/mL) were prepared in the mobile phase and infused with a syringe pump which was coupled with a T-piece to the HPLC pump and the TurboIonSpray® interface of the mass spectrometer. The flow rate of the syringe pump was 0.02 mL/min, the flow rate of the HPLC pump 1.0 mL/min. Abundant peaks corresponding to the protonated molecules $[M+H]^+$ with m/z 399 and m/z 371 were observed in the positive ion mass spectrum of sunitinib and *N*-desethyl sunitinib.

MS/MS product ion mass spectra were obtained on collision-induced dissociation of the protonated molecules of sunitinib (m/z 399) and N-desethyl sunitinib (m/z 371) by scanning Q3. Fig. 1 shows the product ion spectra optimized for the most intensive fragments. The most intensive fragment for sunitinib and N-desethyl sunitinib was m/z 283. For the internal standard the transition m/z 404 → m/z 283.

3.2. Validation

The present study was conducted in accordance with the U.S. Food and Drug Administration (FDA) guidance for industry (bioanalytical method validation) [20] and the European Medicines Agency (EMA) guideline on validation of bioanalytical methods [21], which provide assistance in developing bioanalytical method validation information to yield reliable results that can be safely interpreted in human clinical pharmacology, bioavailability, and bioequivalence studies requiring pharmacokinetic evaluation. The present developed and validated LC–MS/MS method meets all criteria suggested by GLP, FDA [20] and EMA [21] guidelines.

3.2.1. Assay specificity

Based on the analysis of drug-free human plasma (n = 12), matrix components did not interfere with sunitinib, N-desethyl sunitinib and the internal standard (d_5-sunitinib) at their retention times and over the concentration range described herein. Fig. 2 shows typical MRM chromatograms for a blank plasma sample (A), a blank plasma sample spiked with sunitinib and N-desethyl sunitinib (at LLOQ = 0.06 ng/mL) (B), and a plasma sample from a healthy volunteer 384 h after the administration of sunitinib (sunitinib: 1.15 ng/mL and N-desethyl sunitinib: 3.24 ng/mL) (C).

3.2.2. Linearity of calibration curves, precision and accuracy of calibration standards and the LLOQ

The linear regression of the peak area ratios versus concentrations was fitted over the concentration range of 0.060–100 ng/mL for sunitinib and N-desethyl sunitinib in human plasma. The mean linear regression equation of the calibration curves generated during the validation was: $y = -0.0039\ (\pm 0.00146) + 0.3910\ (\pm 0.1125)\ x$ for sunitinib and $y = -0.0017\ (\pm 0.0012) + 0.2948\ (\pm 0.0564)\ x$ for N-desethyl sunitinib, where y represents the ratio of sunitinib or N-desethyl sunitinib peak area to that of the internal standard, and x represents the plasma concentration of sunitinib or N-desethyl sunitinib. Excellent linearity was obtained in the validated concentration range. The correlation coefficients of the weighted calibration curves were ≥0.999 for sunitinib and N-desethyl sunitinib.

The inter-day precision and accuracy (relative error) of the back-calculated calibration standards of sunitinib in human plasma ranged from 0.9 to 5.8% and from −2.5 to 2.1%, respectively. N-desethyl sunitinib inter-day precision and accuracy (relative error) of the back-calculated calibration standards ranged from 0.5 to 4.9% and from −2.4 to 1.9%, respectively. The inter-day precision and relative error obtained at the LLOQ were 2.0% and 1.0% for sunitinib and 1.1% and 0.7% for N-desethyl sunitinib. Using 0.1 mL plasma, the lower limit of quantification for sunitinib and N-desethyl sunitinib was 0.060 ng/mL.

3.2.3. Precision and accuracy

Table 2 summarizes the intra- and inter-day precision and relative error for sunitinib and N-desethyl sunitinib, evaluated by assaying the SQC samples. The inter-day precision of the spiked quality control standards for sunitinib in human plasma ranged from 1.6 to 6.1% with a relative error between 0.2 and 9.1%. The intra-day precision and relative error of the spiked quality control standards of sunitinib in human plasma ranged from 0.4 to 6.4% and 0.0 to 7.9%. The inter-day precision of the spiked quality control standards for N-desethyl sunitinib in human plasma ranged from 1.1 to 5.3% with a relative error between −0.1 and 6.2%. The intra-day precision and relative error of the spiked quality control standards of N-desethyl sunitinib in human plasma ranged from 1.1 to 2.4% and −0.7 to 8.8%. The obtained results were within the acceptance criteria of no more than 20% deviation at LLOQ and no more than 15% deviation for spiked quality control standards above LLOQ.

3.2.4. Recovery

The mean absolute extraction recovery of sunitinib and N-desethyl sunitinib over the whole concentration range was determined as 86.2 ± 6.4% and 84.8 ± 5.0%, respectively. The mean absolute recovery of the internal standard (d_5-sunitinib) at the working concentration was determined as 85.3 ± 0.8%. The simple one-step protein precipitation procedure showed satisfactory recovery for sunitinib and N-desethyl sunitinib in human plasma.

3.2.5. Stability

The results of the 95% confidence interval calculation showed no evidence of instability during chromatography, extraction and sample storage processes for sunitinib and N-desethyl sunitinib in human plasma samples.

3.2.6. Influence of dilution, hemolyzed and lipemic plasma

Diluted (1:5), hemolyzed and lipemic human plasma samples can be analyzed with acceptable precision (sunitinib: 1.1–2.1%; N-desethyl sunitinib: 1.7–5.5%) and accuracy (sunitinib: 93.3–99.5%; N-desethyl sunitinib: 101.2–107.3%). No influence on the determination of sunitinib and N-desethyl sunitinib could be shown for six different batches of human plasma from healthy volunteers.

3.2.7. Influence of different batches of human serum from severely ill patients

No influence on the determination of sunitinib and N-desethyl sunitinib was found for 25 different batches of human serum from 25 severely ill patients (including neonates). None of 21 drugs (aciclovir, anidulafungin, caspofungin, ceftazidime, ciprofloxacin, clindamycin, erythromycin, fluconazole, fosfomycin, ganciclovir, imipenem, linezolid, meropenem, metronidazole, piperacillin, sulbactam, sulfamethoxazole, tigecycline, trimethoprim, vancomycin, voriconazole) received by the patients interfered with sunitinib, N-desethyl sunitinib or the internal standard at their retention times and the concentration range described herein. Since many of these patients had severe organ dysfunction the levels of the drugs and their in part very complex metabolic breakdown and accumulation of metabolites is a true stress test for any assay. The absence of interferences in our assay shows that this assay may not only be used for studies in healthy volunteers, but also in severely sick patients.

3.2.8. Matrix effect

Matrix factors were 1.003 and 0.982 for sunitinib and N-desethyl sunitinib, respectively. These values indicate the absence of a matrix effect in the analytical system. Fig. 3 shows an overlay plot of a chromatogram obtained for post column infusion of sunitinib and N-desethyl sunitinib while injecting a prepared blank human plasma sample and a chromatogram of 5 ng/mL sunitinib and N-desethyl sunitinib in human plasma. No increase or decrease of the signal was observed at and near the retention times of sunitinib and N-desethyl sunitinib, respectively and therefore a matrix effect was excluded.

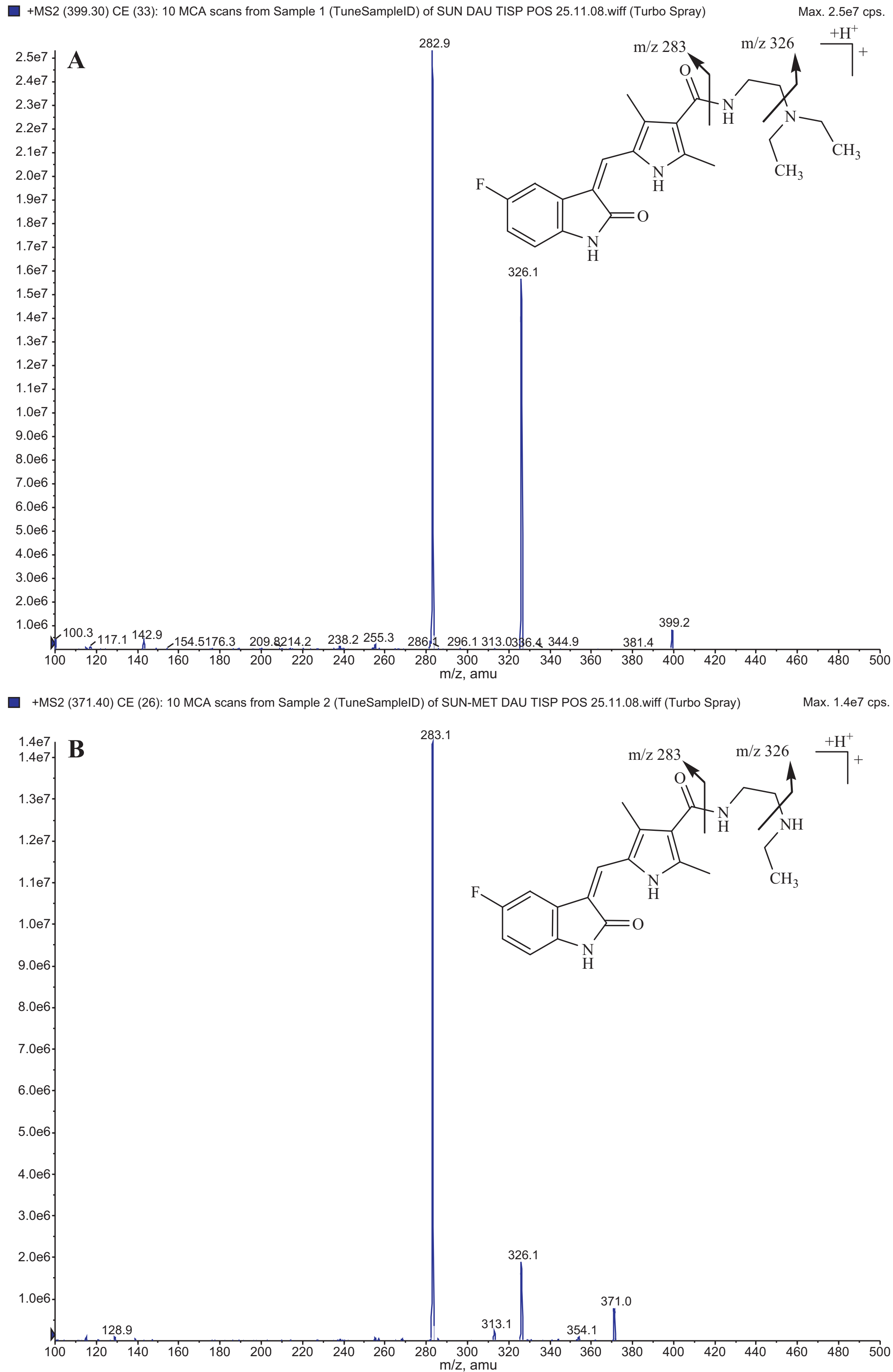

Fig. 1. Product ion mass spectra of sunitinib (A) and *N*-desethyl sunitinib (B).

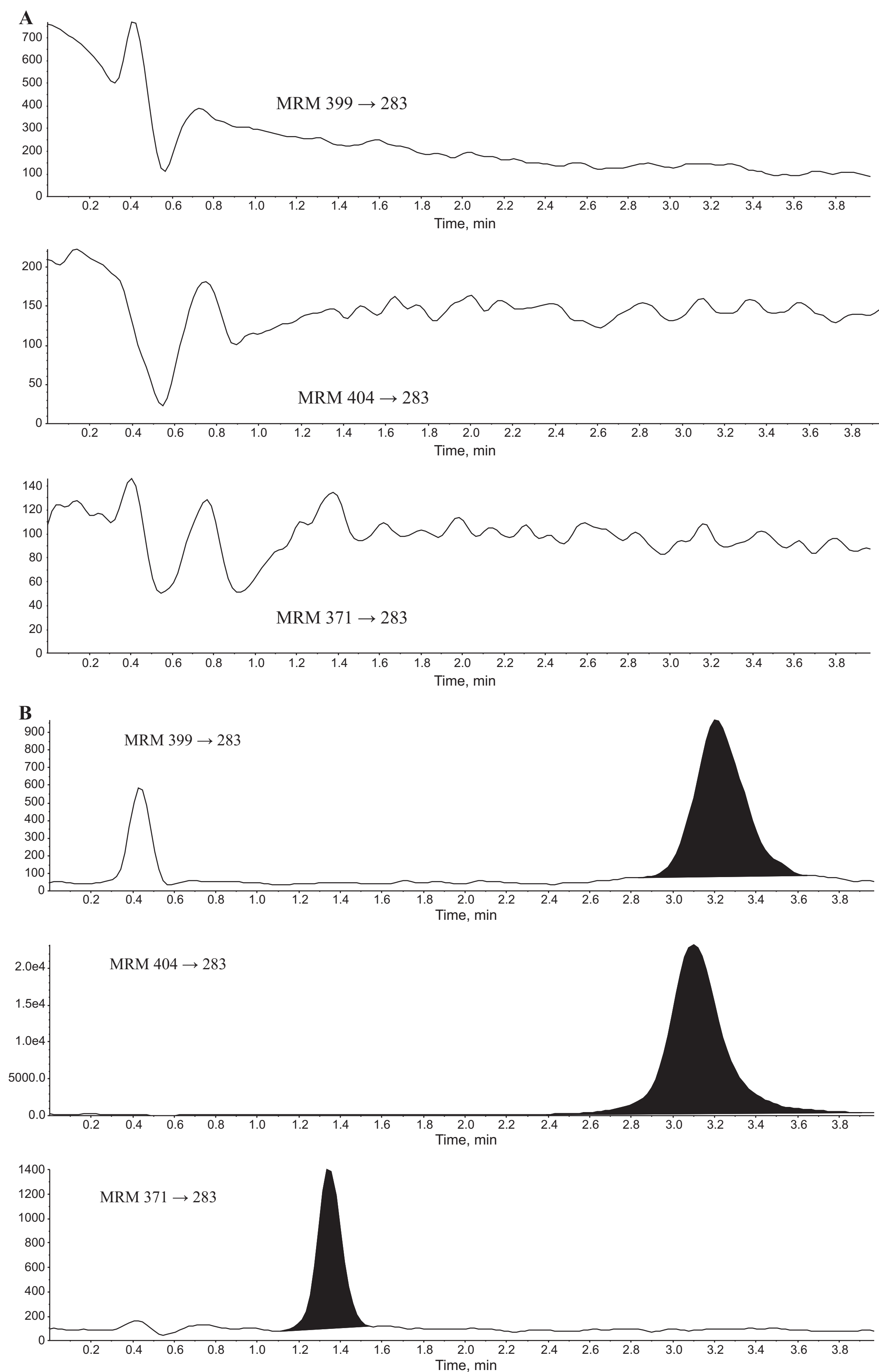

Fig. 2. Representative MRM chromatograms for sunitinib (top), d_5-sunitinib (IS, middle), and *N*-desethyl sunitinib (bottom) in human plasma: (A) a blank plasma sample analyzed after a sample with concentrations of 200 ng/mL for sunitinib and *N*-desethyl sunitinib; (B) a blank plasma sample spiked with sunitinib and *N*-desethyl sunitinib (at LLOQ = 0.06 ng/mL); (C) a plasma sample from a healthy volunteer 384 h after the administration of sunitinib (sunitinib: 1.15 ng/mL and *N*-desethyl sunitinib: 3.24 ng/mL).

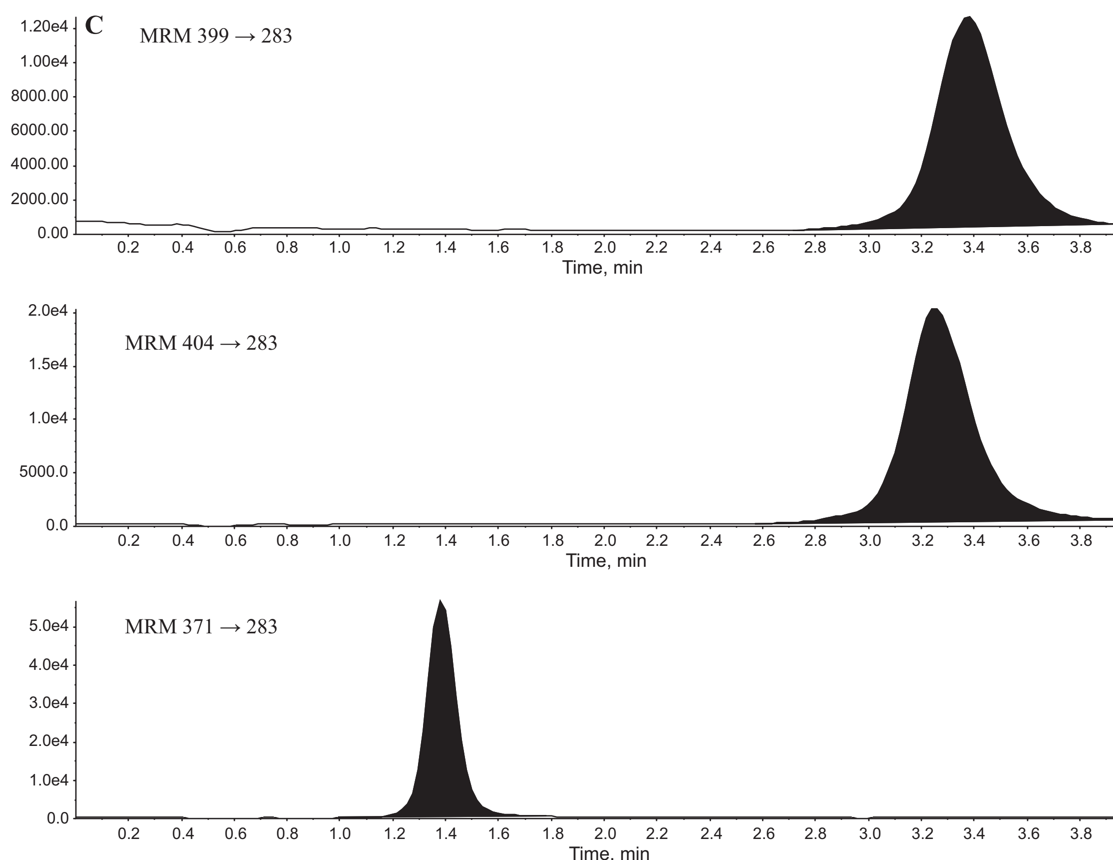

Fig. 2. *Continued*

Table 2
Intra- and inter-day precision and accuracy for the analysis of sunitinib and *N*-desethyl sunitinib in human plasma (five days, five replicates per day).

	Intra-day CV [%]	Intra-day relative error [%]	Inter-day CV [%]	Inter-day relative error [%]
Theoretical concentrations of sunitinib in human plasma				
SQC 1 (100.0 ng/mL)	0.4	1.7	1.7	0.4
SQC 2 (75.00 ng/mL)	0.7	0.6	1.6	0.2
SQC 3 (10.00 ng/mL)	0.8	0.0	1.8	0.8
SQC 4 (0.1500 ng/mL)	4.0	2.0	3.8	0.7
SQC 5 (0.0600 ng/mL)	6.4	7.9	6.1	9.1
Theoretical concentrations of *N*-desethyl sunitinib in human plasma				
SQC 1 (100.0 ng/mL)	1.2	0.6	1.1	−0.1
SQC 2 (75.00 ng/mL)	2.4	−0.7	1.8	1.2
SQC 3 (10.00 ng/mL)	1.1	0.8	1.9	1.9
SQC 4 (0.1500 ng/mL)	2.3	4.9	4.1	3.6
SQC 5 (0.0600 ng/mL)	2.2	8.8	5.3	6.2

3.2.9. Signal to noise

The signal to noise ratio at the LLOQ (0.060 ng/mL) was >5 on all validation days for sunitinib and SU11262, respectively.

3.3. Pharmacokinetic study

Sunitinib and *N*-desethyl sunitinib concentrations of human plasma samples were measured in a total of five sequences in a clinical study that was recently published [25]. The coefficient of correlation of resulting linear regressions was at least 0.9996 for both analytes and all criteria of GLP, FDA [20] and EMA [21] guidelines were met very well.

3.3.1. Sunitinib

The inter-day precision and the analytical recovery of the spiked quality control standards of sunitinib in human plasma ranged from 1.0 to 5.3% and were 100.1% (100.8 ng/mL), 100.9% (74.59 ng/mL), 101.2% (9.619 ng/mL), and 105.6% (0.1484 ng/mL), respectively. Within the set of SQC samples ($n = 15$) analyzed with the batches of study samples, 59 out of 60 SQC samples were within ±15% of their respective nominal value. Therefore, the accuracy and precision of the analysis of the study samples were judged acceptable and all requirements of GLP, FDA [20] and EMA [21] guidelines were met very well.

3.3.2. N-desethyl sunitinib

The inter-day precision and the analytical recovery of the spiked quality control standards of *N*-desethyl sunitinib in human plasma ranged from 1.0 to 5.3% and were 97.8% (109.7 ng/mL), 102.4% (103.9 ng/mL), 101.2% (103.4 ng/mL), and 105.6% (0.1615 ng/mL), respectively. Within the set of SQC samples ($n = 15$) analyzed with the batches of study samples, 59 out of 60 SQC samples were within ±15% of their respective nominal value. Therefore, the accuracy and

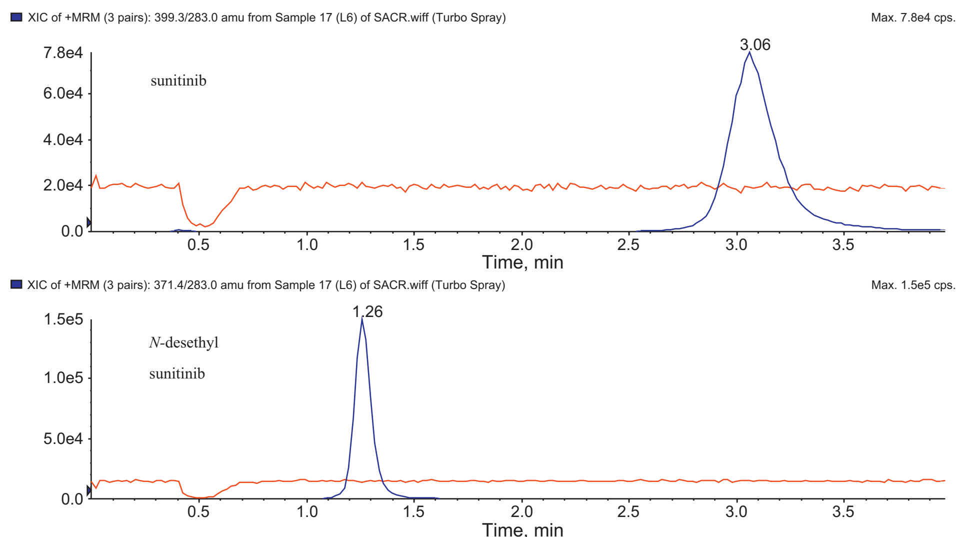

Fig. 3. Overlay plot matrix effect test.

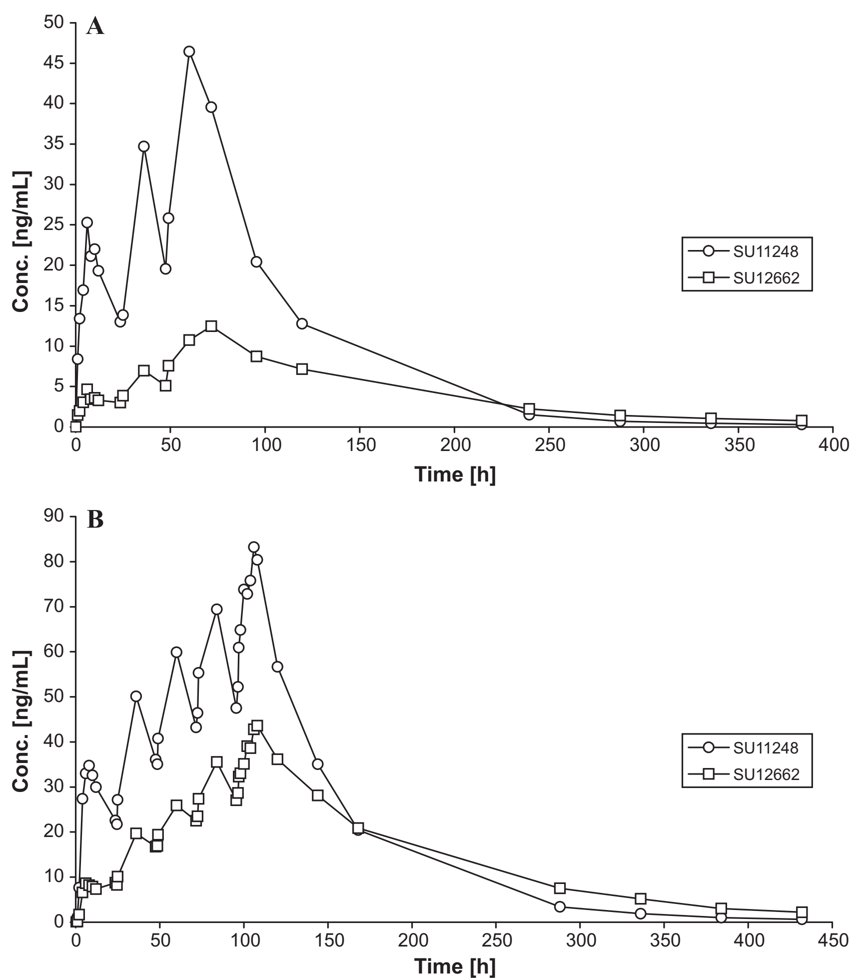

Fig. 4. Plasma profile of sunitinib (SU11248) and *N*-desethyl sunitinib (SU12662) concentration *vs.* time following a 50 mg oral dose of sunitinib once daily for three (A) or five (B) consecutive days of two different healthy volunteers from the pharmacokinetic study [25].

precision of the analysis of the study samples were judged acceptable. Fig. 4 shows as an example the plasma concentration profile of sunitinib and *N*-desethyl sunitinib after oral administration of 50 mg sunitinib once daily for three and five consecutive days to two healthy volunteers.

3.3.3. Incurred samples

The mean precision of the incurred samples of sunitinib and *N*-desethyl sunitinib in human plasma was 2.8% and 2.3%, respectively. These values represent the excellent performance and reproducibility of the developed method. In addition, the EMA

guideline requirement, that the results of the two measurements should be within 20% of the mean for at least 67% of the repeated samples [21], was met during the analysis of the study samples.

3.4. Comparison of the current method to existing methods

Table 1 lists analytically relevant information of nine previously reported methods for the determination of sunitinib and *N*-desethyl sunitinib in comparison with the present LC–MS/MS method. They can be divided into subgroups and a distinction by means of runtime, detection and sample preparation seems to be the most appropriate. Whereas most of the methods developed so far show runtimes below 5 min, the methods by Etienne-Grimaldi et al. [15], Blanchet et al. [14], and Haouala et al. [12] require 10-14 min. As the former two rely on UV-detection the increased runtime is most likely needed to avoid co-elution of other UV-active substances, *e.g.* amino acids, small peptides or co-administered drugs and their metabolites. As the method of Haouala and colleagues was devoted to simultaneous detection of six tyrosine kinase inhibitors, the increased runtime is well explained (sunitinib elutes in this method after approximately 8 min) due to the need to avoid interferences. Obviously, this method becomes very time consuming in clinical trials, where interest relies on one special (in this case late eluting) tyrosine kinase inhibitor and where high sample numbers have to be analyzed or instrument availability is limited.

Although Haouala et al. used MS/MS- instead of UV-detection, only an LLOQ of 1 ng/mL was reached. The presented method exceeds the sensitivity by a factor of about 17 times reaching an LLOQ of 0.06 ng/mL. Compared with the LC–MS and LC–MS/MS methods the LC–UV methods in general show – not too surprisingly – a lower sensitivity, lower selectivity and longer runtimes. Usually a sample volume of at least 0.5 mL is required and no isotope-labeled internal-standards can be used when UV detection is applied. Though LC–UV methods provide enough sensitivity for therapeutic drug monitoring where multiple receptor tyrosin kinases are inhibited in concentrations of 50-100 ng/mL [8], LC–MS/MS is the analytical method of choice when sample volume is low, *e.g.* when several measurements out of one sample have to be performed or when sunitinib and *N*-desethyl sunitinib have to be detected in complex matrices like tissues. LC–MS/MS is also essential when detailed knowledge of the elimination profile of sunitinib and *N*-desethyl sunitinib is to be studied and when samples are to be taken for a very long time after drug administration where concentrations in plasma are extremely low. All methods listed in Table 1 applying MS- or MS/MS-detection use electrospray ionization in positive mode. In comparison to Bello et al. [10] and Minkin et al. [13], representing by today the most sensitive methods, the LLOQ of the present method provides an LLOQ more sensitive by a factor of 1.7 or 3.3.

Taking preparation of plasma samples into account all methods listed in Table 1 either use liquid–liquid extraction or protein precipitation. Whereas liquid–liquid extraction is recognized as a well established procedure only three recent reports by Haouala et al., Zhou et al., and Honeywell et al. use protein precipitation for sample preparation, *i.e.* by means of acetonitrile [12,26] or methanol containing 0.1% acetic acid [17]. However, in case of tissue samples like tumor or brain homogenates other preparation techniques like liquid–liquid or solid-phase extraction may replace protein precipitation as the method of choice [17]. The present method, using protein precipitation by acetonitrile, was successfully used to analyze plasma samples from a clinical study in healthy volunteers [25], and had a fast and convenient sample workup followed by specific, sensitive, fast and reliable quantitation by mass spectrometry.

In addition, two analytical methods by Britten et al. [27] and Fiedler et al. [28] should be noted that are not listed in Table 1. Both report LC–MS/MS measurements of sunitinib and *N*-desethyl sunitinib with an LLOQ of ca. 0.1 ng/mL, but lack any further information about sample preparation procedures or quality data.

4. Conclusions

The developed and validated LC–MS/MS assay for sunitinib and *N*-desethyl sunitinib in human plasma is simple, fast, reliable, sensitive, precise and accurate. The method employs acetonitrile protein precipitation, which allows quantification of sunitinib and *N*-desethyl sunitinib in human plasma for concentrations ranging from 0.06 ng/mL to 100.0 ng/mL using 0.1 mL of plasma. The principal advantage of the LC–MS/MS method described in this paper is the simultaneous achievement of high absolute recovery, high sensitivity, high inter-day precision and high analytical recovery for LLOQ, as well as excellent linearity with a short runtime of only 4.0 min. These characteristics make the method suitable for the precise and accurate measurement of low concentrations of sunitinib and *N*-desethyl sunitinib in the context of pharmacokinetic studies.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2011.02.006.

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