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High-throughput quantification of the anti-leukemia drug STI571 (Gleevec™) and its main metabolite (CGP 74588) in human plasma using liquid chromatography–tandem mass spectrometry

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Abstract

The signal transduction inhibitor STI571 (formerly known as CGP 57148B) or Gleevec™ received fast track approval by the US Food and Drug Administration (FDA) for treatment of chronic myeloid leukemia (CML). STI571 is a revolutionary and promising new oral therapy for CML, which functions at the molecular level with high specificity. The dramatic improvement in efficacy compared to existing treatments prompted an equally profound increase in the pace of development of Gleevec™. The duration from first dose in man to completion of the New Drug Application (NDA) filing was approximately 2.6 years. In order to support all pharmacokinetics studies with sufficient speed to meet various target dates, a semi-automated procedure using protein precipitation was developed and validated. A Tomtec Quadra 96 (Model 320) and a protein precipitation step in a 96-well plate format were utilized. A Sciex API 3000 triple quadrupole mass spectrometer with an atmospheric pressure chemical ionization interface operated in positive ion mode was used for detection. The method proved to be rugged and allowed the simultaneous quantification of STI571 and its main metabolite (CGP 74588) in human plasma. Herein, assay development, validation, and representative concentration–time profiles obtained from clinical studies are presented.

Keywords: STI571; Gleevec; CGP 74588

1. Introduction

Gleevec™ or imatinib mesylate, designated chemically as 4-[(4-methyl-1-piperazinyl)methyl]-*N*-[4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-phenyl]benzamide methanesulfonate, is a protein-tyrosine kinase (PTK) inhibitor which potently inhibits the Abelson (Abl) tyrosine kinase in in vitro [1–3] and in vivo studies [4–11].

Protein-tyrosine kinases (PTKs) are enzymes that have the ability to transfer the terminal phosphate of an adenosine triphosphate (ATP) molecule to a protein substrate. PTKs are critical modulators of cellular signal-transduction pathways, which mediate cell proliferation, differentiation, and communication [12,13]. If for any reason these signaling proteins are subjected to oncogenic mutation(s), a cellular deregu-

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lation may occur, yielding an imbalance between cell division, cell growth, and cell death (apoptosis). Hence, PTKs have emerged as important therapeutic targets for intervention in cancer [14].

The Philadelphia (Ph) chromosome is the consequence of a reciprocal translocation between chromosomes 9 and 22 yielding a fusion oncoprotein referred to as Bcr-Abl (~210 000 Dalton). This molecular consequence leads to an elevated catalytic activity of Bcr-Abl resulting in a resistance to apoptosis, cell transformation, and malignancy. A cytogenetic hallmark of chronic myeloid leukemia (CML), a clonal hematopoetic stem cell disorder, is the Ph chromosome and high activity of Bcr-Abl tyrosine kinase [15–17]. The clinical chemistry manifestation of CML is elevated levels of white blood cells (e.g. $>20\times10^9$/l) and in some patients increase in platelet counts (e.g. $>450\times10^9$/l). Therapeutic options for CML includes allogeneic stem cell transplantation, interferon-alpha treatment, and chemotherapy with hydroxyurea or busulfan [6,18,19]. Allogeneic stem cell transplantation requires the availability of a suitable donor and presents a risk of mortality in older patients. Chemotherapy based methods often do not provide a cure, present toxic side effects, and lead to intolerability and/or resistance to the treatment. In addition, none of the agents used for CML is known to target the underlying cause of the disease.

Recently, FDA approved Gleevec™ (STI571) in a record time [20] for the treatment of patients at any of the three stages of CML: myeloid blast crisis, accelerated phase, and chronic phase after failure of interferon-alpha therapy. Gleevec™ has been referred to as a milestone for the drug development in cancer and an ideal targeted drug at the molecular level [20,21]. With high specificity, it competitively inhibits the binding of ATP to the kinase activation domain of Bcr-Abl (Fig. 1) [22].

For example, Fig. 2 shows the hematologic response starting within only 2 weeks after the initiation of Gleevec™ therapy (500 mg per day). The plot depicts the white blood cell count (WBC) versus the duration (in days) of treatment with Gleevec™ [8]. These patients were in the chronic phase, whom treatment with interferon-alpha had failed. In Fig. 2, each line corresponds to a WBC for an individual patient. The middle horizontal line refers to the upper limit of a normal WBC.

In recent years, a number of laboratories have reported the use of high-throughput bioanalytical procedures using liquid chromatography–tandem mass spectrometry (LC–MS–MS) [23–36]. Herein, we describe the validation of a semi-automated protein precipitation (PPT) method using a Tomtec Quadra 96 (Model 320) in a 96-well plate format. This method proved to be rugged and allowed the simultaneous quantification of Gleevec™ and its main metabolite (CGP 74588) in human plasma.

2. Experimental

2.1. Reagents and materials

High purity solvents were purchased from Fisher Scientific (Springfield, NJ, USA). All other chemicals used were commercially available (Aldrich,

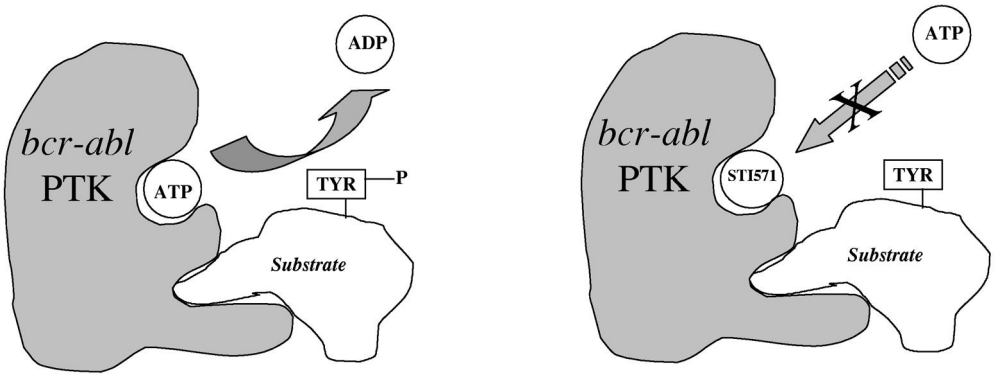

Fig. 1. A simplified representation illustrating the likely mode of action of STI571 or Gleevec™ [41]. STI571 inhibits the binding of adenosine triphosphate (ATP) to the kinase activation domain of Bcr-Abl oncoprotein.

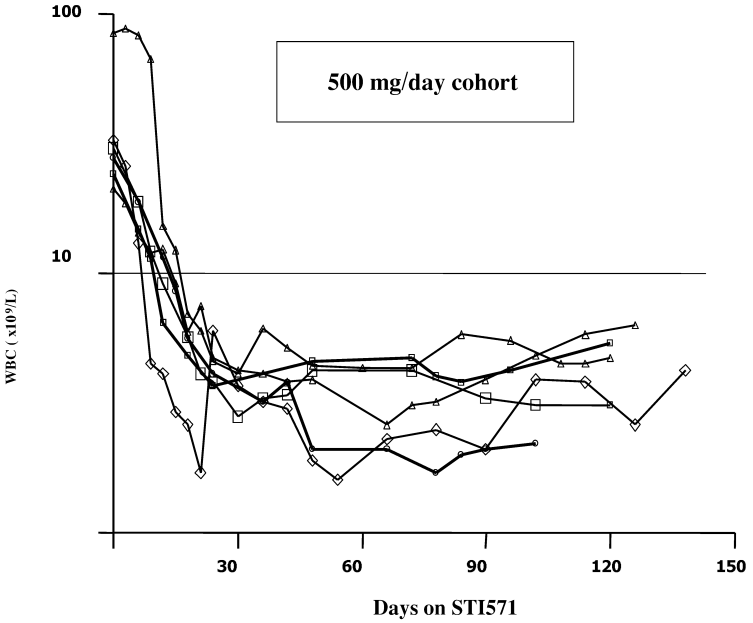

Fig. 2. Hematologic response starting within only 2 weeks after the initiation of STI571 therapy (500 mg per day). The plot depicts the white blood cell count (WBC) versus the duration (in days) of treatment with STI571 [8]. These patients were in the chronic phase, whom treatment with interferon-alpha had failed. Each line corresponds to a WBC for an individual patient. The middle horizontal line indicates the upper limit of a normal WBC (Copyright© 2001 Massachusetts Medical Society).

Milwaukee, WI, USA or Sigma, St. Louis, MO, USA) and of analytical grade. Blank human plasma samples (anticoagulant: disodium EDTA) were obtained from Biological Specialty Corporation (Colmar, PA, USA). STI571 ($C_{30}H_{35}N_7SO_4$ as methanesulfonic acid salt), CGP 74588 ($C_{29}H_{33}N_7SO_4$ as methanesulfonic acid salt), and the internal standard, STI571-D_8 ($C_{29}H_{23}D_8N_7O$), were synthesized at Novartis Pharmaceuticals Corporation (Basel, Switzerland and East Hanover, NJ, USA). The molecular structures of STI571, CGP 74588, and the internal standard are shown in Fig. 3.

2.2. Mass spectrometry

A Sciex API 3000 triple quadrupole mass spectrometer (PE-Sciex, Toronto, Canada) with atmospheric pressure chemical ionization (APCI) in positive ion mode was used for detection. The mass spectrometer was operated in the multiple-reaction monitoring (MRM) mode. The APCI source was operated at a temperature of 450 °C with a corona discharge current of 3 μA. Nitrogen was used as the curtain (setting=10), nebulizing (setting=4), and collision gas (setting=9). The collision energy (Q0-RO2) was set at 38.0 eV (laboratory frame). During the MRM experiments, the nitrogen pressure in the second quadrupole was measured at 3.0×10^{-5} Torr (1 Torr=133.322 Pa). The orifice (OR) and ring (RNG) voltages were set at 80 V and 350 V, respectively. Following HPLC separation, the peak areas corresponding to the m/z 494.3→394.3 reaction (dwell-time=500 ms) for STI571 and m/z 480.2→394.3 reaction (dwell-time=500 ms) for CGP 74588 were measured relative to that of the m/z 502.2→394.3 reaction (dwell-time=200 ms) of the internal standard (Fig. 3). Representative spectra for the collision-induced dissociation (CID) of STI571 and the internal standard are shown in Fig. 4. The same product-ion signal (m/z 394.3) was monitored for CGP 74588 (vide supra).

2.3. Data processing

A Power Macintosh G3 workstation was used for data acquisition and processing. Sample Control (version 1.4), TurboQuan (version 1.0), and Microsoft Excel (version 6.0) were used for data process-

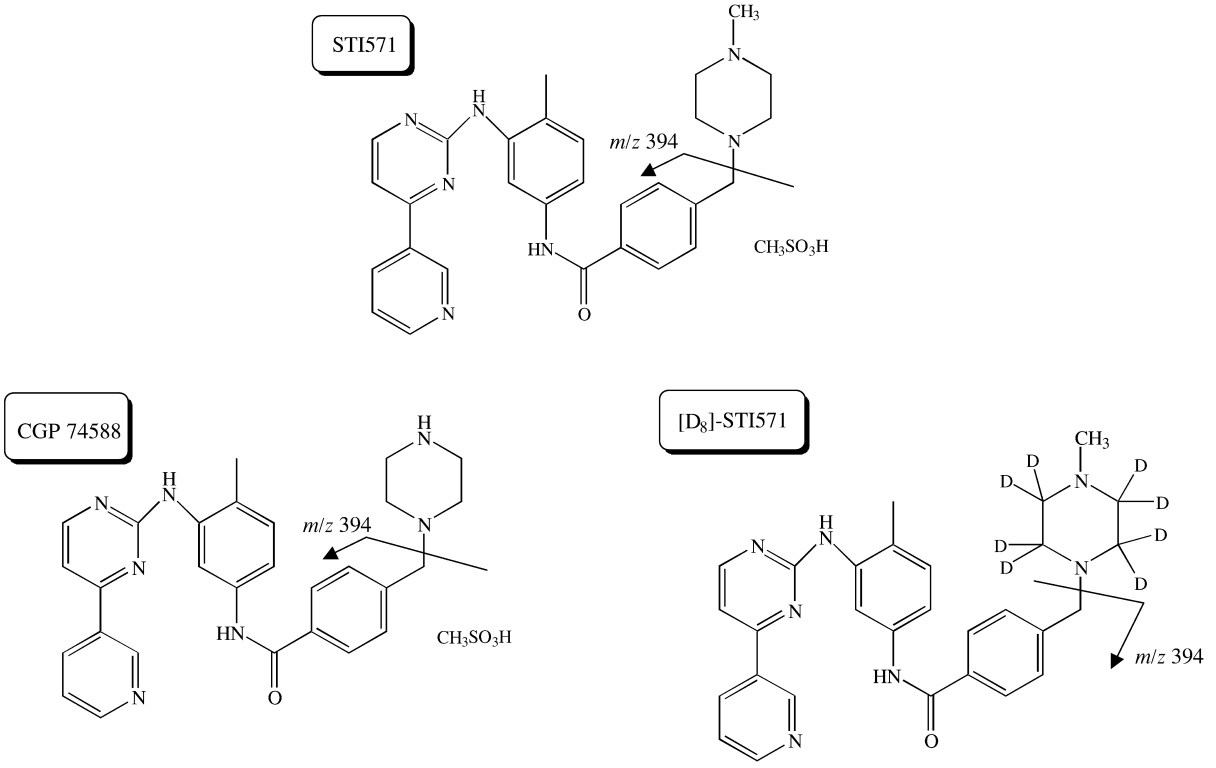

Fig. 3. Structure of STI571, CGP 74588, and the internal standard. Arrows indicate the product-ion selected for the multiple-reaction monitoring experiment.

ing and statistical analysis. A three-point smoothing algorithm was applied to all ion-chromatograms and viewed using MultiView (version 1.4) software. The calibration curve was generated using the results of the calibration samples by linear least-squares regression analysis according to the equation $y = a + bx$, where y was the peak–area ratio of substance to internal standard, x was the concentration of analyte in the calibration sample, a was the intercept, and b was the slope of the regression line. A weighted $(1/x^2)$ linear least-squares regression was used. Subsequently, concentrations of the quality control (QC) samples were calculated from the regression equation of the calibration curve.

2.4. Chromatography

A 50×4.6 mm (I.D.), 3.5-μm, Waters Symmetry Shield-RP_8 column (Milford, MA, USA), packed with C_8 bonded silica particles was used. The pre-column was a Phenomenex (Torrance, CA, USA), C_8 4.0×3.0 mm (I.D.). The LC system consisted of a LC-10AD *VP* pump (Shimadzu, Columbia, MD, USA) and an SCL-10A *VP* controller. The autosampler was a CTC HTS-PAL unit (LEAP Technologies, Carrboro, NC, USA) equipped with a 96-well plate cooling stack set at 15 °C. The HPLC flow-rate was 1.0 ml/min. An on-line degasser, Degassit Model 6324 (MetaChem Technologies, Inc., Torrance, CA, USA), was used. The mobile phase was composed of methanol–water (72:28, v/v) containing 0.05% (by weight) ammonium acetate. Separation was performed at ambient temperature. Sample injection volume was 10 μl. The HPLC and autosampler systems were synchronized via the Power Macintosh G3 workstation (Sample Control 1.4 software).

2.5. Assay procedure

Stock solutions of STI571 and CGP 74588 for

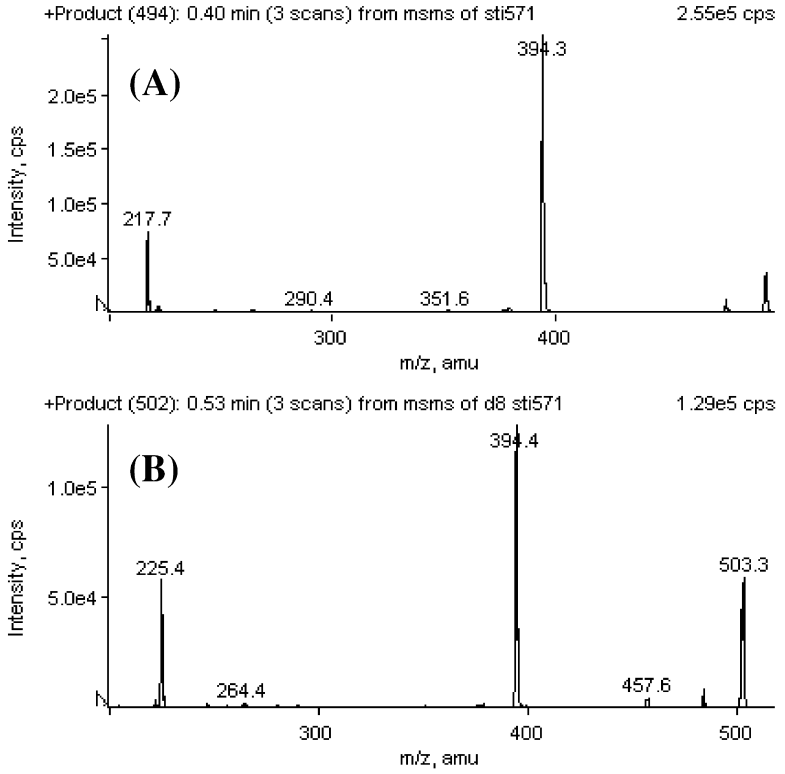

Fig. 4. Representative positive-ion APCI–MS–MS spectra obtained for STI571 (top panel) and the internal standard (bottom panel). CGP 74588 also yielded the same product-ion as STI571 at m/z 394.

calibration standards and QC samples were separately prepared in methanol–water (50:50, v/v) and stored at 4 °C. For the standard curve, the concentrations of STI571 and CGP 74588 (both calculated as the free base) in human plasma were as follows: 4.00, 8.00, 20.0, 100, 1000, 5000, and 10 000 ng/ml. The calibration standards were freshly prepared on each analysis day by adding 50-μl aliquot of the spiking stock solution to 200 μl of blank human plasma. The QC concentrations for STI571 and CGP 74588 in human plasma were 4.00, 8.00, 40.0, 200, 4200, and 8400 ng/ml. Six batches of QC samples with varying lot numbers were prepared as a pool, portioned, and stored at −20 °C pending analysis. A stock solution of the internal standard, STI571-D_8, was prepared in methanol yielding a concentration of 540 ng/ml. Method validation was carried out over a period of 3 days.

In a V-bottom 96-square deep-well block (individual 2.0 ml capacity wells, Microliter Analytical Supplies, Suwanne, GA, USA; part number: 07-7000), 200 μl of human plasma, 50 μl of methanol containing the internal standard (corresponding to 27 ng/sample), and 50 μl of spiking standard solution were added and mixed thoroughly. The calibration curves for STI571 and CGP 74588 were constructed in duplicate. For the QC samples, 200 μl of plasma containing STI571 and CGP74588 and 50 μl the internal standard solution were used. Next, a 250-μl aliquot of acetonitrile was added to precipitate the proteins, using an electronic eight-channel pipette (Brinkman Instruments, Westbury, NY, USA) and vortexed. The wells were capped using a Webseal Mat Closure (Chromacol, Trumbull, CT, USA; part number: WSM-3S) and mixed using a pulsing 96-well plate shaker (Glas-Col, Terre Haute, IN, USA). The speed of the shaker had to be adjusted to prevent any possible leakage between the wells or from the Webseal Mat Closure. Subsequently, the plate was centrifuged (using a refrigerated CENTRA-CL3R or

an IEC CENTRA-7R, International Equipment, Needham Heights, MA, USA) for 10 min at about 1000 *g* (~3000 rpm).

The supernatant (~250 μl) was transferred into a clean V-bottom 96-square deep-well block using a Tomtec Quadra 96 Model 320 unit (software version 2.0, Tomtec, Hamden, CT, USA). Each 96-well block containing the supernatant layer was sealed and transferred to the autosampler, where 10 μl was injected onto the column for LC–APCI–MS–MS analysis.

2.6. Assay validation

2.6.1. Linearity

The linearity of the method was evaluated over the concentration range of 4.00–10 000 ng/ml in human plasma. Calibration standards were freshly prepared in duplicate on each day of validation. The assay acceptance criterion for each back-calculated standard concentration was 15% deviation from the nominal value except the lower limit of quantification (LLOQ) which was set at 20%. The calibration curve had to demonstrate a correlation coefficient of 0.95 or higher. Comparison of the slope, intercept, and correlation coefficient was made for the 3-day validation process.

2.6.2. Intra- and inter-day accuracy and precision

The intra- and inter-day accuracy and precision of the assay were tested by analysis of six QC sample concentrations in replicates of six on three separate days. The precision was expressed as the coefficient of variation (%C.V.). The intra-assay accuracy and precision were calculated as the mean and precision of all individual accuracy of QC samples analyzed during a single analysis run (replicates of six for each QC concentration). The values were calculated for each day of validation separately. The inter-day accuracy and precision were calculated as the mean and the precision over all individual accuracy of QC samples analyzed during the 3 days of validation (replicates of 18 for each QC concentration). The precision acceptance criterion for each QC sample concentration was ≤15% (≤20% for the lowest QC).

2.6.3. Recovery

Recovery of the sample preparation method was assessed by comparison of the peak area obtained from the analysis of neat reference solutions (unprocessed) and of processed human plasma samples. The recoveries of STI571 and CGP 74588 were examined at concentrations of 4.00, 5000, and 10 000 ng/ml ($n=3$ per concentration).

2.6.4. Specificity

Specificity of the assay was demonstrated by obtaining ion-chromatograms for blank pooled human plasma samples as well as blank human plasma spiked with only the internal standard. Blank samples were prepared from six different batches of human plasma and analyzed in duplicate on each day of validation.

2.6.5. Freeze–thaw stability

In freeze–thaw stability studies, samples of STI571 and CGP 74588 (at three concentrations) were subjected to three freeze–thaw cycles and subsequently analyzed in duplicate. Plasma samples were stored at −20 °C for 24 h and thawed unassisted at room temperature. This cycle of thawing and freezing was repeated two more times followed by LC–MS–MS analysis on the third cycle.

2.6.6. Stability

The stability of STI571 and CGP 74588 in human plasma was evaluated over 38 weeks at three concentrations in duplicate. The average time for a complete analytical run of four 96-well plates was about 20 h. Thus, an autosampler stability determination was performed at a 24-h period. The stability of calibration standard stock solutions was also assessed for up to 7 months at 4 °C.

3. Results and discussion

Due to the fast track status of STID571 (Gleevec™) by the US Food and Drug Administra-

tion, a dramatic improvement in throughput of bioanalytical method was warranted. The duration from first dose in man to completion of the New Drug Application (NDA) filing was approximately 2.6 years. In order to complete the pharmacokinetic studies with sufficient speed to meet various target dates, a semi-automated procedure using protein precipitation was developed and validated.

Protein precipitation (PPT) using acetonitrile is a commonly used procedure for the treatment of plasma samples in drug analysis. The simplicity and speed of PPT make it an attractive procedure in high-throughput bioanalysis. Given the current paradigm shift toward automated and robotics systems, we explored the utility of PPT in 96-well format for the quantitative analysis of STI571 and CGP 74588. The current method required a LC–MS–MS run time of about 2.5 min (injection-to-injection cycle). The typical batch sizes were two to four plates a day. The sample preparation step was approximately 2 h per plate. Extensive sample clean up was not needed to ensure column longevity due to the reduced column sample loading. Nonetheless, an optional step of supernatant filtration was tested prior to LC–MS–MS analysis. This step can aid in the removal of the larger particles (e.g. protein aggregates, particle sizes $\geq$10 μm) that may otherwise render technical difficulties during analysis. Filters such as 3M Empore™ (part number: 6060; 3M, Minneapolis, MN, USA) or UNI-FILTER GF/B glass fiber (part number: 7700-1803; Whatman, Clifton, NJ, USA) 96-well plates are among a number of options available for protein filtration.

During the analysis of in vivo samples, the absence of adequate chromatography (i.e. co-elution of analyte and possible metabolites) coupled to MS detection may result in interference, which is not easily detected during the validation process. Recently, Jemal and Xia [37] demonstrated the possibility of in-source dissociation of biotransformation products such as phase II metabolites (e.g. glucuronide conjugate of the parent drug) yielding the parent compound. Thus, during the analysis of post-dose biological samples, a clear understanding of the identity and abundance of in vivo biotransformation products is important. In this case, we did not observe any false positives in terms of post-dose STI571 or CGP 74588 concentrations, which could stem from the in-source dissociation of the above compounds yielding identical MS–MS transitions. The current assay was used to support several major clinical studies and completion of regulatory submission of STI571.

3.1. Linearity

The linearity and reproducibility of calibration curves for STI571 and CGP 74588 in human plasma was acceptable between 4.00 and 10 000 ng/ml. The mean correlation coefficients obtained for STI571 and CGP 74588 over a 3-day validation period were 0.999 and 0.998, respectively. Intercept values were typically less than 0.0026 for both analytes. The mean ($n=3$) calibration slopes for STI571 and CGP 74588 were 0.01250 ± 0.00133 and 0.00817 ± 0.00009, respectively. Tables 1 and 2 summarize the calibration curve results for STI571 and CGP 74588, respectively. Mean accuracy of standard calibration samples for STI571 covering the concentration of 4.00–10 000 ng/ml ranged between 98.0% to 102% with C.V.s of 1.66–5.97%. Mean accuracy of standard calibration samples for CGP 74588 covering the concentration of 4.00–10 000 ng/ml ranged between 98.0 and 104% with C.V.s of 1.58–7.04%.

3.2. Intra- and inter-day results

QC samples covering the anticipated dynamic concentration range of the method, a minimum of six replicates for each QC concentration (Tables 3 and

Table 1
Mean accuracy and precision of back-calculated concentrations of calibration samples for STI571

Nominal concentration (ng/ml)	Mean ($n=6$)	SD	C.V. (%)	Accuracy (%)
4.00	3.97	0.163	4.11	99.3
8.00	8.12	0.485	5.97	102
20.0	19.8	0.628	3.17	99.0
100	102	4.14	4.06	102
1000	1022	17.0	1.66	102
5000	4898	344	7.02	98.0
10 000	9806	387	3.95	98.1

Table 2
Mean accuracy and precision of back-calculated concentrations of calibration samples for CGP 74588

Nominal concentration (ng/ml)	Mean ($n=6$)	SD	C.V. (%)	Accuracy (%)
4.00	4.15	0.0668	1.61	104
8.00	7.48	0.346	4.63	93.5
20.0	19.6	1.38	7.04	98.0
100	99.7	5.71	5.73	99.7
1000	1031	16.3	1.58	103
5000	5081	245	4.82	102
10 000	10 035	315	3.14	100

Table 3
Accuracy and precision of STI571 QC samples in human plasma

Nominal concentration (ng/ml)	Intra-day			Inter-day		
				Mean ($n=18$) accuracy (%)	SD (%)	C.V. (%)
4.00						
Mean ($n=6$) accuracy (%)	102	103	106	104	5.87	5.64
SD (%)	8.59	4.20	3.49			
C.V. (%)	8.42	4.08	3.29			
8.00						
Mean ($n=6$) accuracy (%)	98.4	101	104	101	4.85	4.80
SD (%)	2.57	3.63	6.29			
C.V. (%)	2.61	3.59	6.05			
40.0						
Mean ($n=6$) accuracy (%)	101	104	109	105	5.34	5.09
SD (%)	2.57	2.07	7.22			
C.V. (%)	2.54	1.99	6.62			
200						
Mean ($n=6$) accuracy (%)	107	105	112	108	4.92	4.56
SD (%)	5.55	2.56	3.52			
C.V. (%)	5.19	2.35	2.56			
4200						
Mean ($n=6$) accuracy (%)	104	100	106	103	5.20	5.05
SD (%)	6.50	3.80	4.08			
C.V. (%)	6.25	3.80	3.85			
8400						
Mean ($n=6$) accuracy (%)	103	95.2	98.4	98.9	4.59	4.64
SD (%)	3.06	4.26	2.40			
C.V. (%)	2.97	4.47	2.44			

4), were analyzed on each validation day. The mean intra-day accuracy of QC samples at LLOQ for STI571 ranged 102–106%. The C.V.s were between 3.29 and 8.42%. The mean intra-day accuracy of QC samples above LLOQ for STI571 ranged 95.2–112%. The C.V.s were between 1.99 and 6.62%. The mean intra-day accuracy of QC samples at LLOQ for CGP 74588 ranged 96.3–101%. The C.V.s were between 3.41 and 4.44%. The mean intra-day accuracy of QC samples above LLOQ for CGP 74588 ranged 91.6–111%. The C.V.s were between 1.40 and 6.98%.

The mean ($n=18$) inter-day accuracy values for QC samples obtained for STI571 and CGP 74588 at LLOQ were 104 and 99.2%, respectively. The C.V. values for STI571 and CGP 74588 were 5.64 and 4.19%, respectively. At above LLOQ, STI571 QCs yielded mean accuracy values between 98.9 and 108% with C.V.s of 4.56–5.09%. Likewise, the mean accuracy values for CGP 74588 at above LLOQ spanned from 94.1 to 106% with C.V. values of 3.75–5.07%. Overall, the assay exhibited excellent accuracy and reproducibility within the tested concentration range.

3.3. Recovery

Recovery of STI571 and CGP 74588 using this procedure, compared to an aqueous standard solution at 4.00, 5000, and 10 000 ng/ml was investigated in duplicate, at each concentration. The mean recovery values for STI571 and CGP74588 were >85 and $>90\%$, respectively.

Table 4
Accuracy and precision of CGP 74588 QC samples in human plasma

Nominal concentration (ng/ml)	Intra-day			Inter-day		
				Mean ($n=18$) accuracy (%)	SD (%)	C.V. (%)
4.00						
Mean ($n=6$) accuracy (%)	101	101	96.3	99.2	4.16	4.19
SD (%)	3.44	3.66	4.28			
C.V. (%)	3.41	3.62	4.44			
8.00						
Mean ($n=6$) accuracy (%)	95.8	95.0	91.6	94.1	3.53	3.75
SD (%)	3.38	4.13	1.28			
C.V. (%)	3.53	4.35	1.40			
40.0						
Mean ($n=6$) accuracy (%)	99.2	99.4	96.8	98.5	4.99	5.07
SD (%)	3.37	4.78	6.76			
C.V. (%)	3.40	4.81	6.98			
200						
Mean ($n=6$) accuracy (%)	105	103	111	106	5.25	4.95
SD (%)	5.04	3.97	3.83			
C.V. (%)	4.80	3.85	3.45			
4200						
Mean ($n=6$) accuracy (%)	106	103	109	106	5.10	4.81
SD (%)	5.62	4.00	4.72			
C.V. (%)	5.30	3.88	4.33			
8400						
Mean ($n=6$) accuracy (%)	105	100	103	103	5.04	4.89
SD (%)	6.97	4.08	2.52			
C.V. (%)	6.64	4.08	2.45			

3.4. Specificity

The specificity of the method was examined by analyzing blank human plasma extract (Fig. 5) and an extract spiked only with the internal standard (Fig. 6). As shown in Fig. 5, no significant interference in the blank plasma traces was seen from endogenous substances in drug-free human plasma at the retention time of the either analytes. Fig. 6 shows the lack of interference from the internal standard to the MRM channels of STI571 and CGP 74588. Fig. 7 depicts a representative ion-chromatogram for the LLOQ (4.00 ng/ml) of the calibration curve. Excellent sensitivity was observed for 10 μl injection volume corresponding to ca. 40 pg on-column.

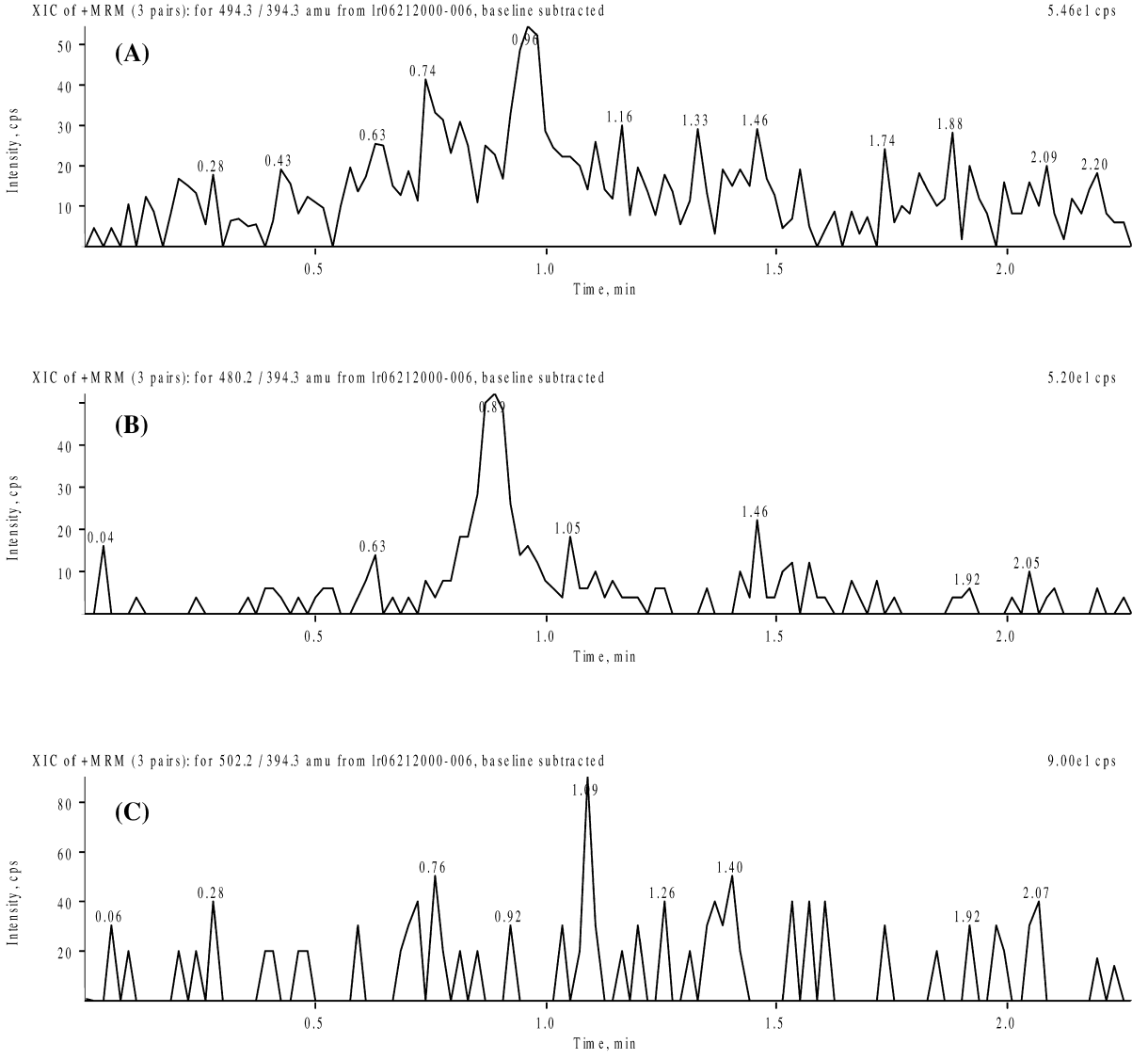

Fig. 5. LC–APCI–MS–MS ion-chromatograms resulting from the analysis of blank (drug and internal standard free) human plasma for STI571 (panel A), CGP 74588 (panel B), and STI571-D_8 (panel C).

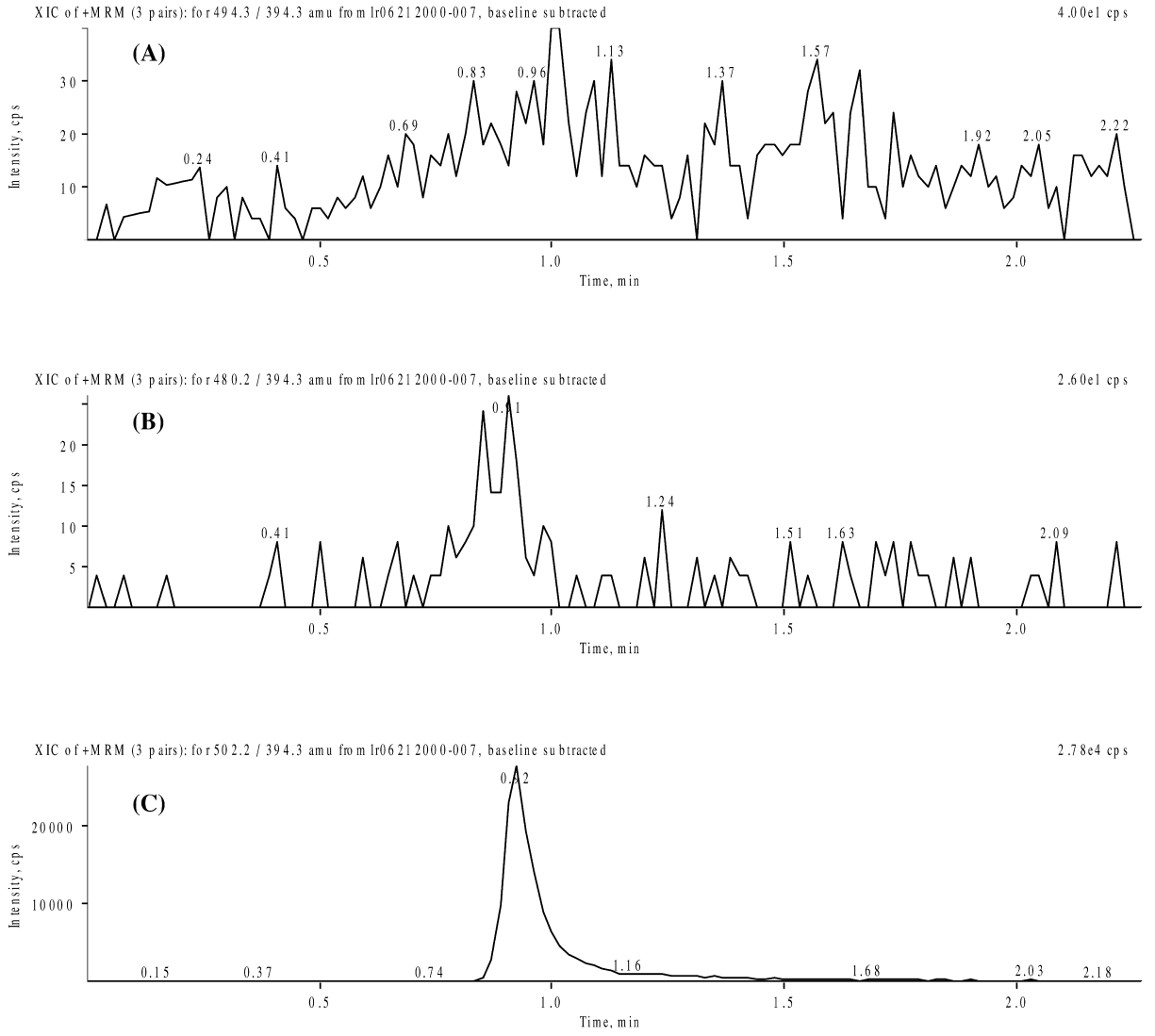

Fig. 6. LC–APCI–MS–MS ion-chromatograms resulting from the analysis of blank (drug-free spiked with STI571-D_8) human plasma for STI571 (panel A), CGP 74588 (panel B), and STI571-D_8 (panel C).

3.5. Freeze–thaw, room temperature, and storage stability

Tables 5 and 6 show a summary of the freeze–thaw and storage stability studies for STI571 and CGP 74588, respectively. In the freeze–thaw stability study, samples were subjected to three freeze–thaw cycles and subsequently analyzed in duplicate. Briefly, plasma samples were stored at −20 °C for 24 h and thawed unassisted at room temperature. This cycle of thawing and freezing was repeated two more times followed by LC–MS–MS analysis on the third cycle. No evidence of sample degradation was observed at concentrations of 2.0, 50, and 1000 ng/ml for either analytes in human plasma. Furthermore, STI571 and CGP 74588 exhibited excellent room temperature (benchtop) stability for at least up to 72 h in human plasma. Long term storage stability

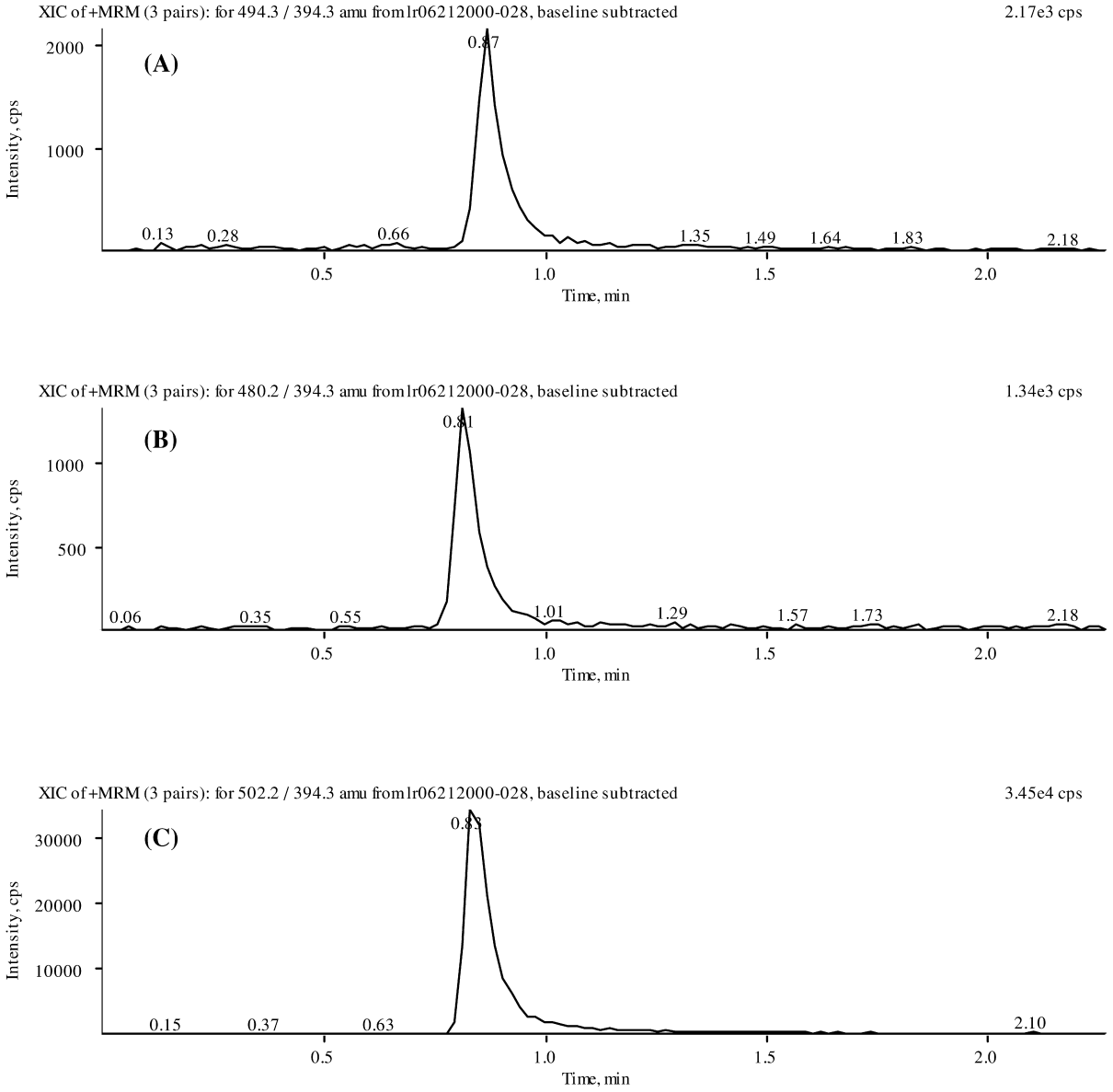

Fig. 7. Representative LC–APCI–MS–MS ion-chromatograms resulting from the analysis of 4.00 ng/ml (LLOQ) of STI571 and CGP 74588 spiked with the internal standard (27 ng/sample). The method did not require evaporation and reconstitution of samples. The injection volume of supernatant was 10 μl. Excellent sensitivity was obtained for ca. 40 pg/ml of on-column injection for each analyte. Panels A, B, and C correspond to the STI571, CGP 74588, and the internal standard, respectively.

Table 5
Summary of results obtained from freeze–thaw, room temperature, and storage stability studies for STI571 in human plasma

Storage period/ conditions	Nominal concentration (ng/ml)	Relative recovery (%)		
		First replicate	Second replicate	Mean
3 Freeze–thaw cycles	2	101	93.4	97.2
	50	103	100	102
	1000	94.5	98.2	96.4
0 h, 25 °C	2	84.7	88.3	86.5
	50	99.0	95.9	97.5
	1000	95.0	94.9	95.0
3 h, 25 °C	2	95.5	95.1	95.3
	50	98.8	97.9	98.4
	1000	91.9	94.9	93.4
24 h, 25 °C	2	95.0	91.6	93.3
	50	93.7	101.2	97.5
	1000	88.0	95.2	91.6
72 h, 25 °C	2	95.4	100	97.9
	50	105.1	98.4	101.8
	1000	98.1	97.5	97.8
1 week, −20 °C	2	92.9	95.1	94.0
	50	108	100	104.1
	1000	106	88.4	97.1
6 weeks, −20 °C	2	87.3	98.3	92.8
	50	100	94.9	97.6
	1000	103	94.4	98.8
38 week, −20 °C	2	115	102	109
	50	102	102	102
	1000	88.6	89.1	88.9

Recovery values were expressed in percent of the nominal concentration (ng/ml).

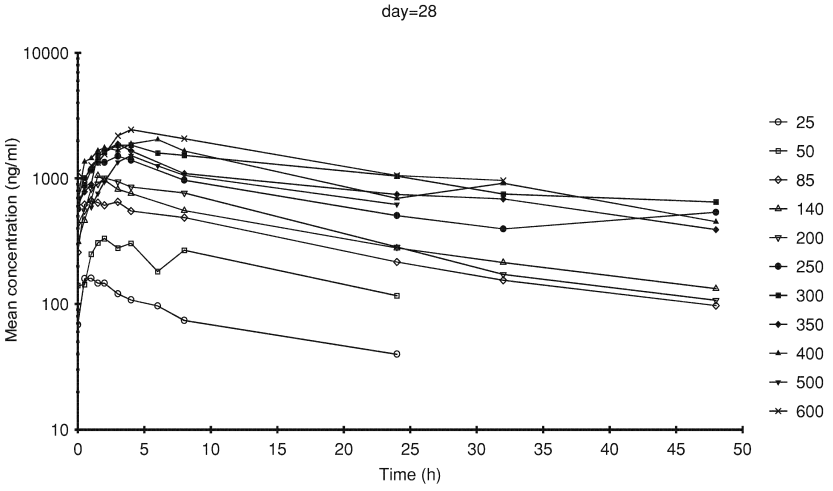

Fig. 8. Mean plasma concentration–time profile for patients subsequent to a daily oral administration of STI571 (free base) for 28 consecutive days. The sampling period was up to 48 h post-dose. The dose regimen was escalated from 25 to 600 mg/day till a favourable hematologic response was observed.

Table 6
Summary of results obtained from freeze–thaw, room temperature, and storage stability studies for CGP 74588 in human plasma

Storage period/ conditions	Nominal concentration (ng/ml)	Relative recovery (%)		
		First replicate	Second replicate	Mean
3 Freeze–thaw cycles	2	103	117	110
	50	100	95.1	97.6
	1000	96.3	98.7	97.5
0 h, 25 °C	2	107	101	104
	50	85.2	97.5	91.4
	1000	90.2	93.9	92.1
3 h, 25 °C	2	126	98.7	112
	50	101	96.5	99.0
	1000	96.8	96.3	96.6
24 h, 25 °C	2	92.7	105	99.0
	50	92.5	87.8	90.2
	1000	88.2	90.6	89.4
72 h, 25 °C	2	117	116	117
	50	92.0	90,4	91.2
	1000	97.9	97.2	97.6
1 weeks, −20 °C	2	108	88.1	97.9
	50	110	88.3	98.9
	1000	97.3	81.7	89.5
6 weeks, −20 °C	2	97.3	81.2	89.3
	50	102	87.4	94.9
	1000	116	110	113
38 weeks, −20 °C	2	115	119	117
	50	95.1	94.7	94.9
	1000	87.3	95.7	91.5

Recovery values were expressed in percent of the nominal concentration (ng/ml).

for up to 38 weeks did not lead to any discernible loss of STI571 or CGP 74588 in human plasma (Tables 5 and 6). In addition, a 24-h autosampler stability study did not point to any STI571 and CGP 74588 degradation in the human plasma sample extracts. The stock solutions of both analytes were also found to be stable for at least 7 months at 4 °C.

3.6. Assay application

The current assay was utilized successfully in support of several major clinical studies. The HPLC column was changed after the analysis of every three to four 96-well plates. Although the chromatographic capacity factor was only about 1.0, no significant ion-suppression was observed at the lower concentrations. This is not surprising since ion-suppression has a more dramatic manifestation on electrospray ionization than APCI [38–40].

Although we were able to detect ($S/N>3$) STI571 and CGP 74588 at 250 pg/ml (data not shown), the method was validated with a LLOQ of 4.00 ng/ml. The relatively high plasma concentrations of analytes following an effective therapeutic dose (e.g. 400–600 mg/day) did not warrant attempts to perform validation at a greater sensitivity. Fig. 8 depicts representative *mean* (number of subjects varied between two and seven for each dose profile) concentration–time profiles for patients subsequent to a daily oral administration of Gleevec™ for 28 consecutive days (steady state). The dose regimen was escalated from 25 to 600 mg/day (Fig. 8) till a favourable hematologic response was observed. Fig. 9 illustrates a representative concentration–time profile for STI571 (400 mg daily oral dose; time points taken on days 1 and 9) and CGP 74588 in plasma obtained from a human subject with Philadelphia chromosome-positive CML in myeloid blast crisis

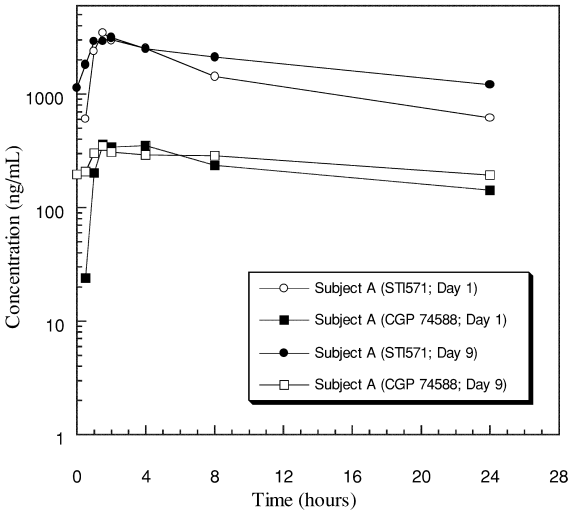

Fig. 9. A representative concentration–time profile for STI571 (400 mg daily oral dose) and CGP 74588 in plasma obtained from a human subject with Philadelphia chromosome-positive CML in myeloid blast crisis (terminal phase with median survival of 3–6 months). The sampling period was up to 24 h post-dose. The post-dose samples corresponded to days 1 and 9.

(terminal phase with median survival of 3–6 months). Based on a number of clinical trials, the plasma AUC for the metabolite is about 15% of the AUC for STI571. A detailed account of the pharmacokinetics of STI571 will be reported separately.

4. Conclusions

We have demonstrated a method for the quantification of STI571 and its main metabolite, CGP 74588, in human plasma using a semi-automated PPT method and a relatively rapid LC–APCI–MS–MS analysis. The assay exhibited an excellent linearity from 4.00 to 10 000 ng/ml in human plasma. The current method was utilized for the analysis of thousands of clinical samples. Furthermore, the method was routinely amenable to analysis of STI571 and CGP 74588 in cerebral spinal fluids (CSF), gastrointestinal stromal tumor (GIST) biopsy specimens, and toxicokinetic studies (data not shown). The simplicity and speed of PPT make it an attractive procedure in high-throughput bioanalysis. Although the number of published semi-automated or fully automated PPT procedures has been small thus far, in comparison with the automated solid-phase extraction protocols, it is envisaged that this type of assay will become more routinely implemented in the near future.

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