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Liquid chromatographic method for detection and quantitation of STI-571 and its main metabolite *N*-desmethyl-STI in plasma, urine, cerebrospinal fluid, culture medium and cell preparations

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Abstract

An isocratic online-enrichment HPLC-assay was developed allowing for the simple and fast separation and quantitation of STI-571 and its main metabolite *N*-desmethyl-STI (*N*-DesM-STI) in plasma, urine, cerebrospinal fluid (CSF), culture media and cell preparations in various concentrations using UV-detection at 260 nm. The analytical procedure consists of an online concentration of STI-571 and *N*-DesM-STI in the HPLC system followed by the elution on a ZirChrom-PBD analytical column. Time of analysis is 40 min including the enrichment time of 5 min. The detection limit is 10 ng/ml in plasma, CSF, culture medium (RPMI) and 25 ng/ml in urine for both STI-571 and *N*-DesM-STI. The intra-day precision, as expressed by the coefficient of variation (CV), in plasma samples ranges between 1.74 and 8.60% for STI-571 and 1.45 and 8.87% for *N*-DesM-STI. The corresponding values for urine measurements are 2.17–7.54% (STI-571) and 1.31–9.51% (*N*-DesM-STI). The inter-day precision analyzed over a 7-month time period was 8.31% (STI-571) or 6.88% (*N*-DesM-STI) and 16.45% (STI-571) or 14.83% (*N*-DesM-STI) for a concentration of 1000 ng/ml in plasma and 750 ng/ml in urine, respectively. Moreover, we demonstrate that with an alternative, but more time and labor consuming sample preparation and the implementation of electrochemical detection, a detection limit <10 ng/ml can be achieved. The method described was used to perform pharmacokinetic measurements of STI-571 and *N*-desmethyl-STI in patient samples and for kinetic measurements of intracellular STI-571 and *N*-DesM-STI following in vitro incubation.

Keywords: STI-571; *N*-Desmethyl-STI

1. Introduction

STI-571 (Imatinib Mesylate, Glivec™), a 4-[(4-methyl-1-piperazinyl)methyl]-*N*-[4-[1–3]-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-phenyl] benzamide methane–sulfonate derivative, acts as an inhibitor of the abl tyrosine kinase, platelet derived growth factor receptor (PDGFR), stem cell factor receptor (c-kit, steel factor receptor, CD117) and ARG tyrosine kinases. The specific blockade of the bcr–abl oncoprotein has been associated with significant antileukemic activity in patients with chronic myeloid leukemia (CML) and Philadelphia positive acute lymphatic leukemia (Ph^+ALL). STI-571 exerts its inhibitory action by blocking the ATP-binding site present in the abl domain of the bcr–abl tyrosine kinase thus resulting in continuous suppression of the bcr–abl tyrosine kinase activity. The reconstitution of the apoptotic pathways in bcr–abl positive cells results in specific anti-leukemic activity [1–14].

STI-571 is usually administered orally at a dose of 400, 600 or 800 mg o.d. (once daily) in different stages of CML or Ph^+ALL with dose modifications according to adverse events and hematologic response. In contrast to other

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cytotoxic drugs, it appears that a close concentration/effect ratio can be defined for STI-571 [15]. Due to its specific mode of action, an effective drug concentration obtained in vitro can be extrapolated to the clinical setting [12]. This assumption is supported by recent studies describing a close correlation between clinical response in CML patients and the STI-571 dose administered in a range from 200 to 600 mg o.d. [16]. Despite the increasing number of clinical studies showing striking effects of STI-571 in different entities, only few reports focused on the pharmacokinetic characteristics of STI-571 in humans [17–21].

Since the major metabolite of STI-571, *N*-desmethyl-STI (*N*-DesM-STI) can be predicted to have comparable intrinsic activity, there is a rationale to measure both STI-571 and *N*-DesM-STI concentrations in different cellular and extracellular compartments in studies of cellular drug resistance to STI-571 and to determine the drugs penetration into specific tissue compartments, e.g. the cerebrospinal fluid (CSF) [22,23]. A sensitive, simple, reliable, inexpensive and broadly applicable detection and quantification method for both STI-571 and its main metabolite *N*-DesM-STI is a basic prerequisite for elucidating STI-571 pharmacokinetics. To date, only one HPLC method published by Bakhtiar et al. satisfies these demands but requires high-end laboratory equipment including liquid chromatography–tandem mass spectrometry, not readily available in most HPLC laboratories [24]. Moreover, this method was validated neither for the analysis of intracellular STI-571- and *N*-DesM-STI concentration nor for the measurement of these substances in urine, CSF and RPMI-medium. Since STI-571 has a limited solubility in pure aqueous solutions, results could conceivably differ between samples with a high proportion of protein-bound STI-571 (e.g. 95% in plasma) and those in aqueous solutions. Moreover, its not clear whether the intracellular uptake of the substance used as internal standard in this method is comparable to that of STI-571.

Other HPLC methods published so far, have either been not described in detail or are limited by detection limits too high for these applications [17,25,26]. In order to fulfill all the demands needed for pharmacokinetic studies, we established and extensively validated a sensitive, simple and fast HPLC method allowing for the separation and quantitation of STI-571 and its main metabolite *N*-DesM-STI in plasma, urine, CSF, culture media without the use of liquid chromatography–tandem mass spectrometry.

2. Experimental

2.1. Reagents, cell incubation and materials

Solvents with high purity and all other chemicals of analytical grade were purchased from Merck (Darmstadt, Germany). Human EDTA plasma and human urine were derived from healthy volunteers. For the validation of intracellular measurements of STI-571 and *N*-DesM-STI, experiments were performed with the cell lines HL-60, K562 and CCRF purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) GmbH (Braunschweig, Germany). HL-60 cells were grown in RPMI-1640 medium (Biochrom AG, Berlin, Germany) supplemented with penicillin, streptomycin and 10% fetal calf serum (FCS) under standard conditions. Cell cultures were performed at 37 °C and 5% CO_2 atmosphere in easy-flasks, 25 cm^2 (Nalge-Nunc, Denmark, Roskilde).

STI-571 was kindly provided by Novartis Pharma GmbH. STI-571 stock solutions for HPLC standards were dissolved in 100% methanol and stored at −20 °C at a concentration of 1000 μg/ml. Since *N*-DesM-STI is not commercial available it was separated and purified by liquid–liquid extraction from patient urine and subsequently purified by the HPLC method described below. The resulting *N*-DesM-STI solution showed a purity of 99% as tested by analytical HPLC. *N*-DesM-STI authenticity was proved by the similarity of UV-spectra, existence solely in STI-571 treated patient plasma or urine and finally by magnet-resonance spectroscopy (data not shown). *N*-DesM-STI stock solutions for HPLC standards were dissolved like STI-571 in 100% methanol and stored at −20 °C at a concentration of 1000 μg/ml. The chemical structure of STI-571, its main metabolite *N*-DesM-STI and other known metabolites with minor formation, are depicted in Fig. 1.

2.2. HPLC apparatus

The chromatographic system consisted of two Knauer 64 analytical HPLC pumps (Knauer, Berlin, Germany), a Shimadzu UV-spectrometric detector SPD-6A (Shimadzu, Duisburg, Germany) adjusted to a wavelengh of 260 nm, a Merck-Hitachi AS 2000A autosampler and an electric motor driven autoswitch equipped with an Rheodyne valve 7740-001 for online-enrichment switching (Besta-HPLC-Technik, Wilhelmsfeld, Germany), as depicted in Fig. 2. Electrochemical detection were performed on an ESA Colouchem 2 detector (ESA, Chelmsford, USA). Recording, evaluation and quantitation of chromatograms as well as the controlling of valve switching was done by a PC supported program from Raytest (GINA, Straubenhardt, Germany). In a limited number of analyses the authenticity of STI-571 and *N*-DesM-STI was proved by analyzing the UV-spectra using a Waters-diode-array detector 994 (Waters, Milford, USA).

2.3. Chromatography

A ZirChrom HPLC column, 3 μm, PDB-ZrO_2, 3% carbon, 50 mm × 4.6 mm with a precolumn of the same solid phase specifity was used in the analytical part of the system. The system was designed as online-enrichment system with another PDB-ZrO_2 precolumn as enrichment column as depicted in Fig. 2. Flow was set on 0.4 ml/min at room temperature in the analytical part and on 2 ml/min at room

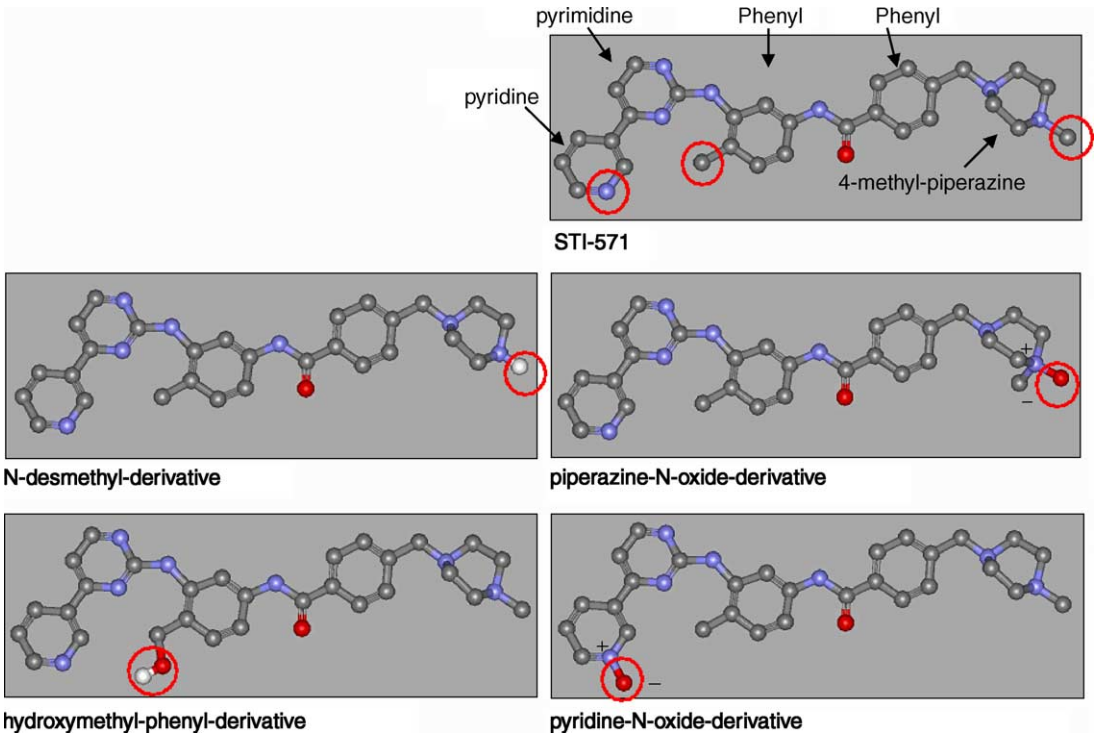

Fig. 1. Chemical structure of STI-571 and STI-571 metabolites—altered groups in the metabolites are marked by circles.

temperature in the enrichment part. The mobile phase B (analytical eluent, eluent B) consisted of 600 ml 0.01 M KH_2PO_4/0.09 M K_2HPO_4 + 400 ml methanol/l (v/v), while the mobile phase A (enrichment eluent, eluent A) was prepared with 450 ml 0.1 M KH_2PO4 + 350 ml, H_2O + 200 ml CH_3OH (v/v). The analysis was performed by UV-detection adjusted at 260 nm or if a sensitivity better than 10 ng/ml were needed by electrochemical detection adjusted to +160 mV for the first ESA cell and +580 mV for the second. The measurement started with a 5 min enrichment period with the enrichment valve in position A, while eluent B was continuously delivered trough the analytical column by pump B. After 5 min enrichment time the enrichment valve was switched to position B and the flow from pump B was redirected trough the enrichment column, eluting STI-571 and *N*-DesM-STI on to the analytical column and

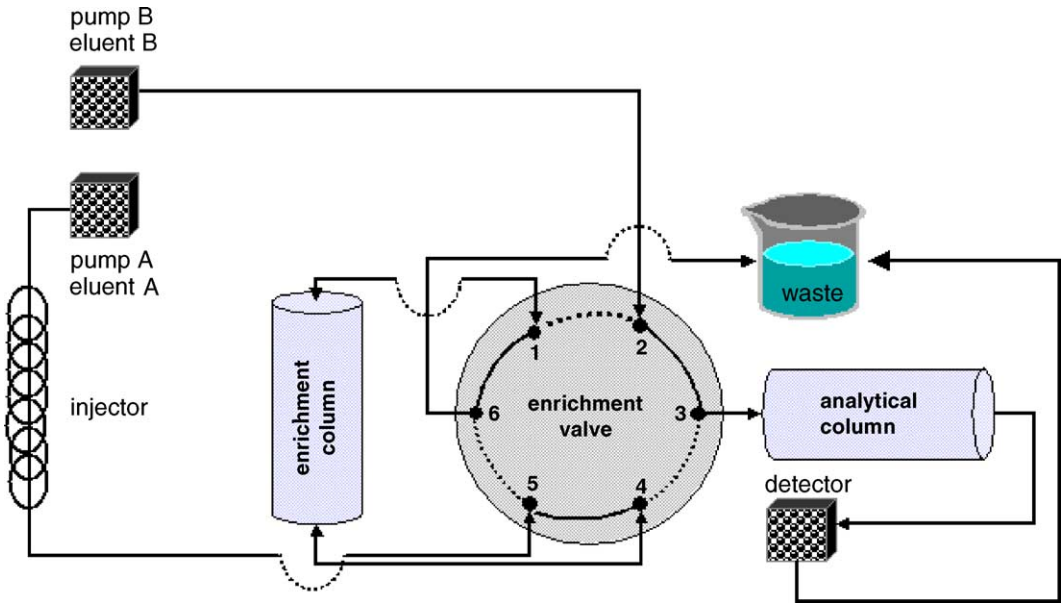

Fig. 2. Schematic valve switching graph for the online-enrichment system.

subsequently to the detector (Fig. 2). After 20 min the precolumn was reequilibrated for 2 min to enrichment conditions and the next sample could be injected at time point 35 min, resulting in a net analytical time of 40 min. Both substances of interest were eluted at this time point with a median retention time (RT) of 9.57 min (range 8.30–12.10) for STI-571 and 29.98 min (range 26.77–35.97) for *N*-DesM-STI, respectively, analyzed over a time period of seven months, 160 injections and multiple column exchanges.

2.4. Quantitation of STI-571 and N-DesM-STI

Quantitation of plasma or protein-containing samples (RPMI-medium, HL-60 cells) was performed using the external standard method by linear regression analysis of 9×10 spiked plasma sample sets with 10, 25, 60, 110, 600, 1100, 4000, 8000 and 20 000 ng/ml for STI-571 and, after authentification of the extracted *N*-DesM-STI samples, with purified *N*-DesM-STI, respectively (in total 90 independent processed samples with nine different concentrations, every concentration 10 times analyzed). Calibration curve for urine or non-protein-containing samples (CSF) was prepared with 7×10 spiked urine sample sets containing 25, 70, 400, 1000, 4250, 8500 and 20,000 ng/ml for STI-571 and *N*-DesM-STI (in total 70 independent processed samples with seven different concentrations, every concentration 10 times analyzed). The calibration data are fitted to a linear, unweighted, forced-through-zero model using the mean of the respective concentration computed by the calibration function of the chromatography software (GINA, Raytest, Straubenhardt, Germany). The resultant linear regression curves were used to calculate the concentrations of the quality control (QC) of STI-571 and *N*-DesM-STI in the respective samples (protein- or non-protein-containing samples).

2.5. Sample preparation

Protein-containing samples (plasma, RPMI-medium) were prepared by adding 30 μl concentrated (85%) perchlorid acid to a 300 μl sample volume. This mixture was agitated with an autoshaker for 10 min. Afterwards 200 μl of the enrichment eluent (eluent A) were added to the sample and mixed for another 10 min. Proteins were then precipitated by centrifugation for 7 min at $4000 \times g$. Of the resulting supernatant 200 μl were injected into the HPLC system for analysis. We tested the stability of STI-571 and *N*-DesM-STI in this solution and found no significant degradation for any of this substances over a time period of 3 days at room temperature (data not shown).

Non-protein samples like CSF or urine were diluted with enrichment eluent A (50/50, v/v) and after 7 min centrifugation at $4000 \times g$, 200 μl were injected without further preparation. As expected this solution was stable for 3 days at room temperature. The same stability of STI-571 and *N*-DesM-STI was observed in non-prepared plasma, RPMI, urine or CSF samples allowing the shipment of samples without the need of freezing. Even in non-centrifuged and subsequently hemolysed blood samples no major change of plasma concentration of STI-571 and *N*-DesM-STI was seen, indicating a distribution ratio of nearly 1:1 between red blood cells and plasma (data not shown).

Sample preparation for detection limits <10 ng/ml using electrochemical analysis was performed in a larger solution volume. To 500 μl of the test specimens 100 μl 1 M NaOH and 3000 μl $CHCL_3$ were added (v/v/v) and agitated for 10 min in glass vials with polytetrafluoroethylene (PTFE) cup septa. The sample was then centrifuged at $4000 \times g$ for 10 min. From the original 3000 μl $CHCL_3$ 2000 μl were drawn and subsequently evaporated to dryness using a common speedvac centrifuge. The residue was reconstituted in 250 μl 100% CH_3OH by shaking the sample for 10 min and 200 μl of the resolved sample were injected into the HPLC.

For intracellular measurement 2×10^7 cells were incubated in 5 ml RPMI at various concentrations of STI-571 and *N*-DesM-STI as indicated below. After an incubation time of 5 h at 37 °C and 5% CO_2, the cells were centrifuged at $1000 \times g$ and the resulting pellets were resolved in 10 ml phosphate buffered saline (PBS) and subsequently pelleted again by centrifugation for 5 min. After discarding the supernatant the sample pellet was prepared for STI-571 and *N*-DesM-STI extraction adding 300 μl isotonic NaCl solution plus 30 μl perchloric acid. This solution was vortexed for 7 min and subsequently 200 μl eluent A was added to the mixture. After further agitation for another 7 min the samples were pelleted by centrifugation at $4000 \times g$ and an 200 μl aliquot of the supernatant was injected into the HPLC enrichment system.

2.6. Method application

The application of the described analytical procedures was examinated on plasma and urine samples from two CML patients after a last dose of 500 and 800 mg/o.d./p.o. (per oral) STI-571 before conditioning therapy and allogeneic bone marrow transplantation. Plasma samples were collected before and after 0.66, 1.25, 2.25, 3.25, 5.25, 6.25, 8.25, 11.25, 13.25, 25.15, 26.15, 48.00 72.00, 96.00, 120.00 and 146.00 h. The length of this sampling period in conjuction with the high sensitivity of the HPLC method allowed a more exact pharmacokinetic calculation especially of the half-life of *N*-DesM-STI, compared with sampling times from one- to the next trough-level, usually 24 h. Urine in these patients was analyzed after 2.00, 5.50, 7.50, 9.00, 12.50, 19.00, 22.00 and 26.10 h after discontinuation of STI-571 therapy. Moreover, we analyzed STI-571 pharmakokinetics in 32 patients with CML after the first application with 400 or 600 mg/o.d./p.o. STI-571 for a sampling period of up to 48 h. Pharmacokinetic parameters were determined by the "Topfit" pharmacokinetic computer program. Additionally, we measured the STI-571 and *N*-DesM-STI CSF concentration in a 56-year-old patient with CML in major

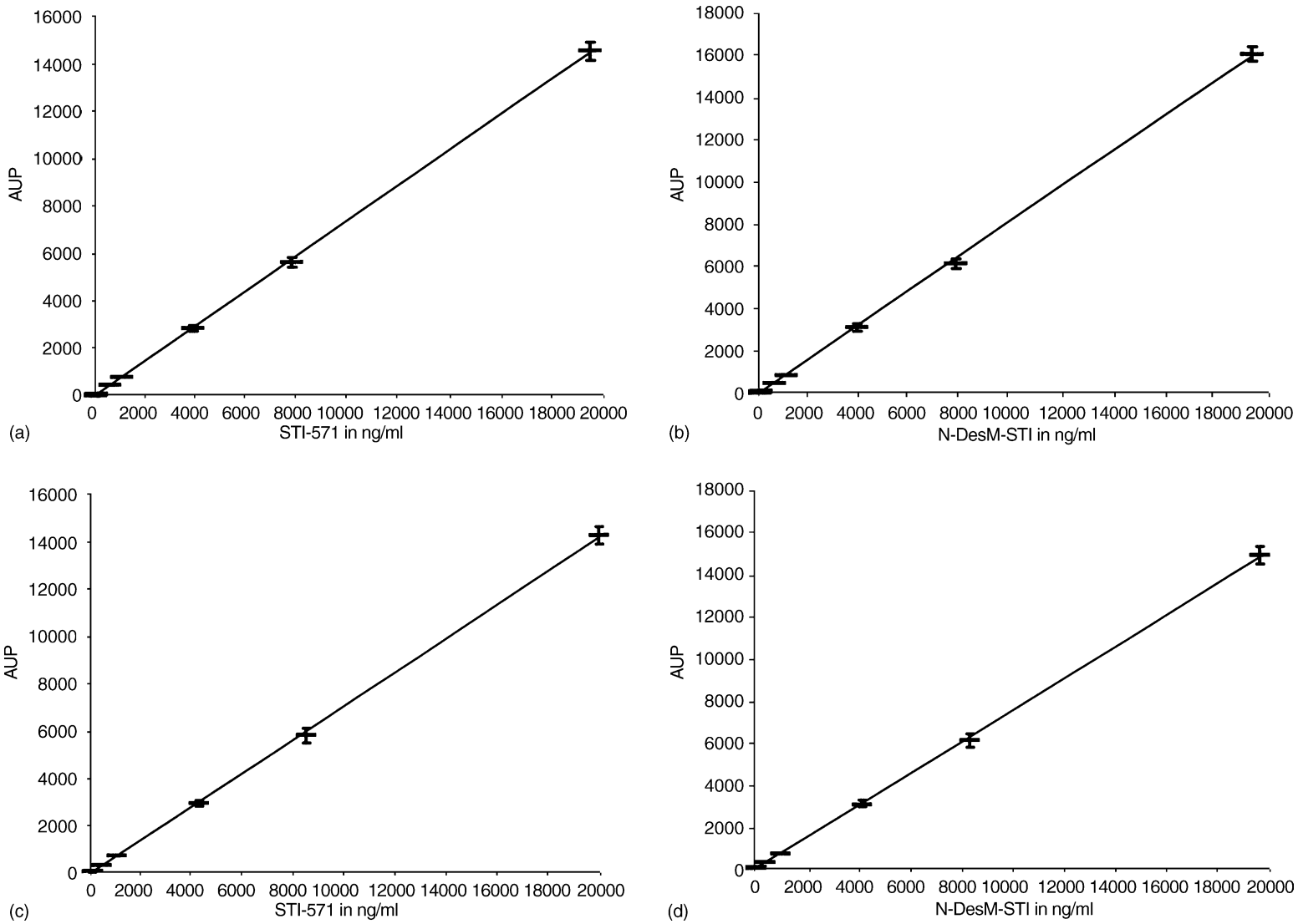

Fig. 3. Regression curves for (a) STI-571 in plasma samples; (b) *N*-DesM-STI in plasma samples; (c) STI-571 in urine samples; (d) *N*-DesM-STI in urine samples.

cytogenetic remission and isolated lymphoid blast crisis in the CSF treated with STI-571 at 200–400 mg/o.d./p.o.

2.7. Pharmacokinetic calculations

The pharmacokinetic analysis of measured concentrations were based on the TOPFIT software program providing an optimized adaption of coefficients of variation between the observed and calculated respective data [26,27].

3. Assay validation

3.1. Linearity

The linearity of the method was evaluated over a concentration range of 10 ng/ml–20 μg/ml in human plasma and of 25 ng/ml–20 μg/ml in human urine, with concentrations as described above (calculated as the free base). One sample was spiked with the appropriate amount of STI-571 and *N*-DesM-STI from stock solution, divided into 10 samples and sample preparation was performed separately for every single probe. The calibration standard was freshly diluted from stock solution every day of validation and accepted if the measured value deviated less than 10% from the nominal expected value for concentrations above 100 ng/ml and less than 15% for concentrations under 100 ng/ml.

3.2. Specifity

Specifity of the assay was tested by analyzing 10 plasma, urine and CSF samples from patients not treated with STI-571 and with 10 plasma and urine samples from healthy volunteers, respectively. Moreover, we analyzed RPMI and three different cell lines for coeluting substances in 10 independent analysis for every sample specimen (HL-60, K562, CCRF cells).

3.3. Recovery in plasma and urine samples

Recovery of the protein- and non-protein-containing sample preparation method was assessed by comparison of the peak area obtained from the analysis of unprocessed methanol standard solutions and processed human plasma and human urine samples with a concentration of 2 μg/ml STI-571 and *N*-DesM-STI, respectively ($n = 10$ for every sample specimen). Recovery for the alternative sample preparation (described earlier) with 500 μl plasma were tested with 10 samples containing 5 ng/ml STI-571 and *N*-DesM-STI.

3.4. Intra- and inter-day precision and accuracy

The intra-assay precision was determined by analyzing 10 independent prepared human plasma quality control or urine QC samples on 1 day and is expressed as the coefficient of variation (CV) of this experiment. The intra-day accuracy was calculated as the ratio of the spiked, as controlled in the parallel prepared methanol standard sample, to the mean measured concentration. These values were calculated for every tested concentration separately over a total evaluation time period of 3 months, while the 10 samples for every concentration point were always analyzed in 1 day.

The inter-assay variance was tested with 22 human plasma QC samples and 12 urine QC samples with nominal 1 μg/ml and 750 ng/ml over a time period of 7 months. Precision is expressed as the CV of all measurements and accuracy as the ratio from the spiked to the mean measured concentration.

Since the amount of CSF was limited, 10 samples with a spiked concentration of 28 ng/ml were evaluated for intra-day precision and accuracy.

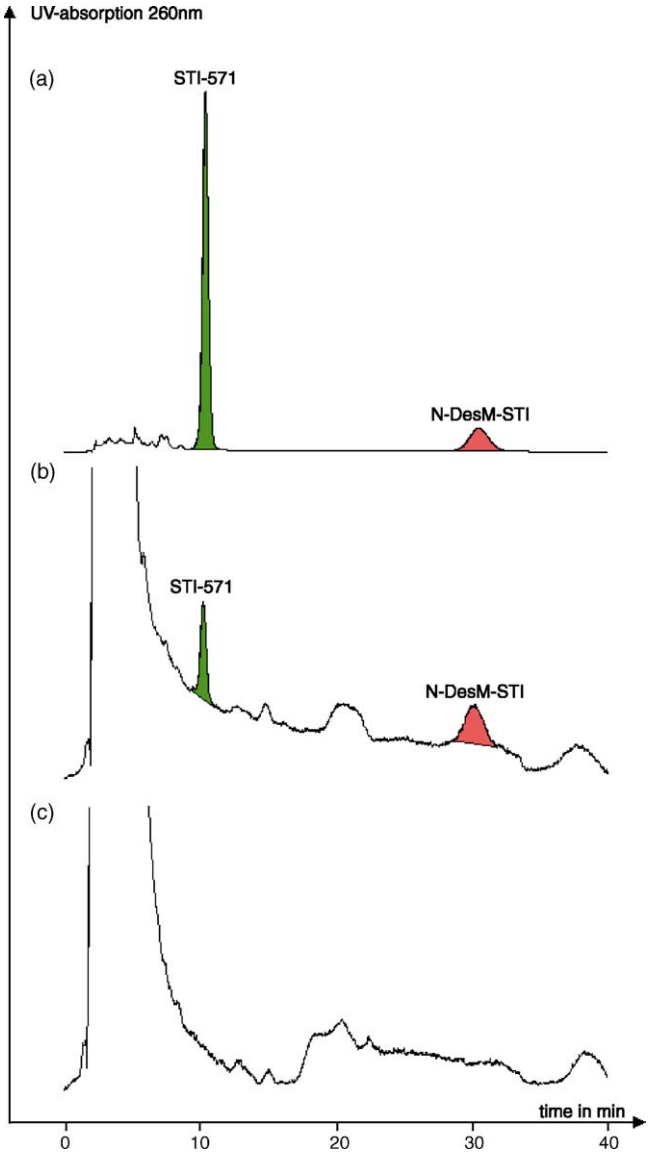

Fig. 4. (a) Patient plasma under STI-571 treatment with 5308 ng/ml STI-571 and 988 ng/ml *N*-DesM-STI; (b) spiked plasma with 10 ng/ml STI-571 and *N*-DesM-STI; (c) blank human plasma (blank and spiked plasma are shown in equal ordinate attenuation).

The precise determination of intra- or inter-day accuracy for intracellular measurements is not practicable, since its is technically not possible to spike cells with a specific concentration of STI-571 or *N*-DesM-STI. The precision of intracellular measurements was calculated from 20 incubation experiments (10 with 6.5 μg/ml and 10 with 200 ng/ml STI-571 and *N*-DesM-STI incubation concentration) using the following way to underline the plausibility of the results. Since STI-571 is metabolized in liver cells mainly via cytochrome P450-3A4 but not in the tested cell lines, the incubation system could be defined as a "closed system" and the sum of STI-571 and *N*-DesM-STI concentrations in all compartments including washing solutions should add up to 100% of the starting concentration. Hence, we added all measured "compartment" concentrations after the incubation period. If the sum of these concentrations added up to 90–110% of the theoretical value, it was highly expectable that the concentration measured in the pellets after centrifugation portrayed the "true" intracellular concentration. This assumption is based on the fact, that the variations of the measurements in RPMI-medium, washing solution and cells add to about 5–15%, which means that the resulting sum of these measurements "has" to add randomly to 85–115% of the expected value.

The problem of determining linearity for intracellular measurements was the same as described above. It was highly unlikely that STI-571 and *N*-DesM-STI have a linear uptake in relation to the extracellular incubation concentration. In fact, we could show that

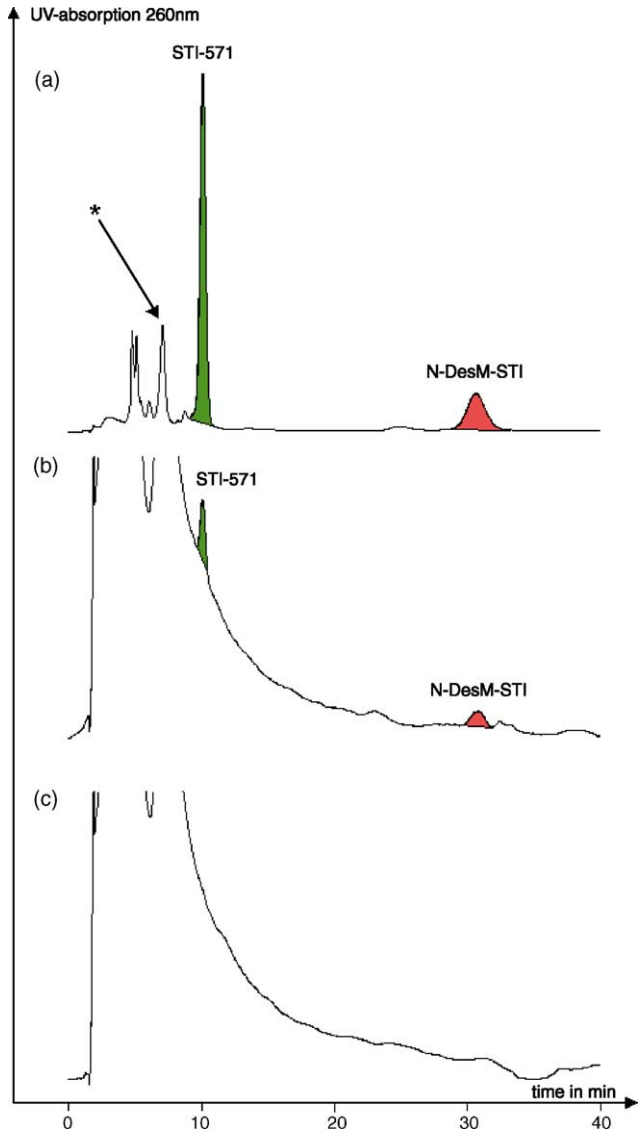

Fig. 5. (a) Patient urine under STI-571 treatment with 9999 ng/ml STI-571 and 3208 ng/ml *N*-DesM-STI; (b) spiked urine with 25 ng/ml STI-571 and *N*-DesM-STI; (c) blank human urine; for (*) see Fig. 7 (blank and spiked urine are shown in equal ordinate attenuation).

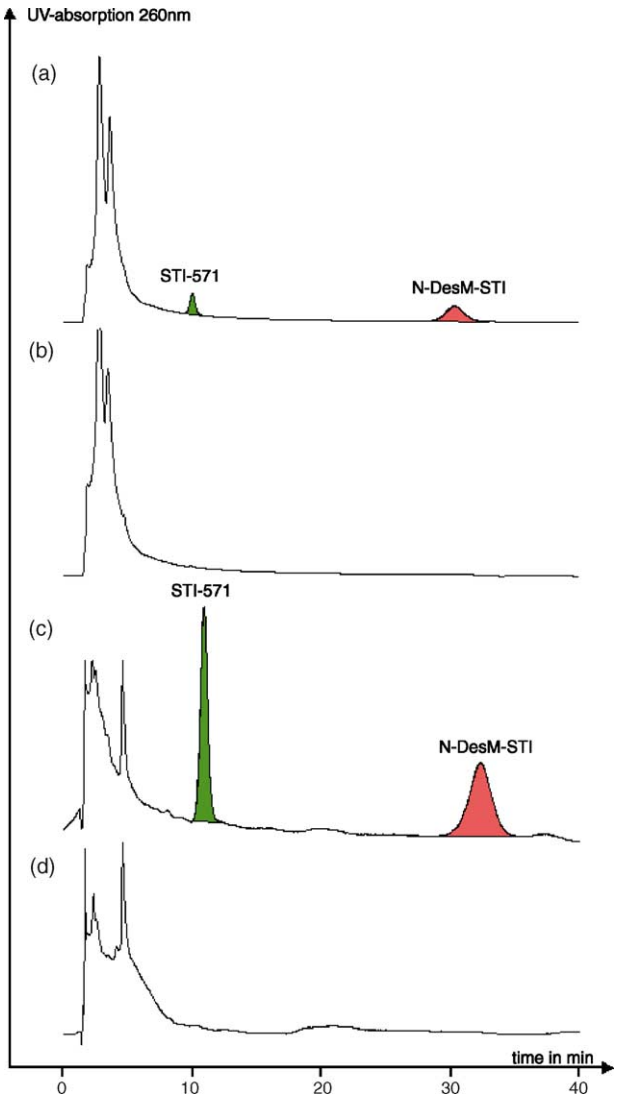

Fig. 6. (a) Intracellular concentration of STI-571 and *N*-DesM-STI in HL-60 cells after 5 h incubation with 200 ng/ml of both substances—aliquot of 7.5 × 10*e*6 cells injected; (b) blank HL-60 cells—aliquot of 7.5 × 10*e*6 cells injected; (c) spiked RPMI with 200 ng/ml STI-571 and *N*-DesM-STI; (d) blank RPMI (spiked or incubated samples are shown in equal ordinate attenuation).

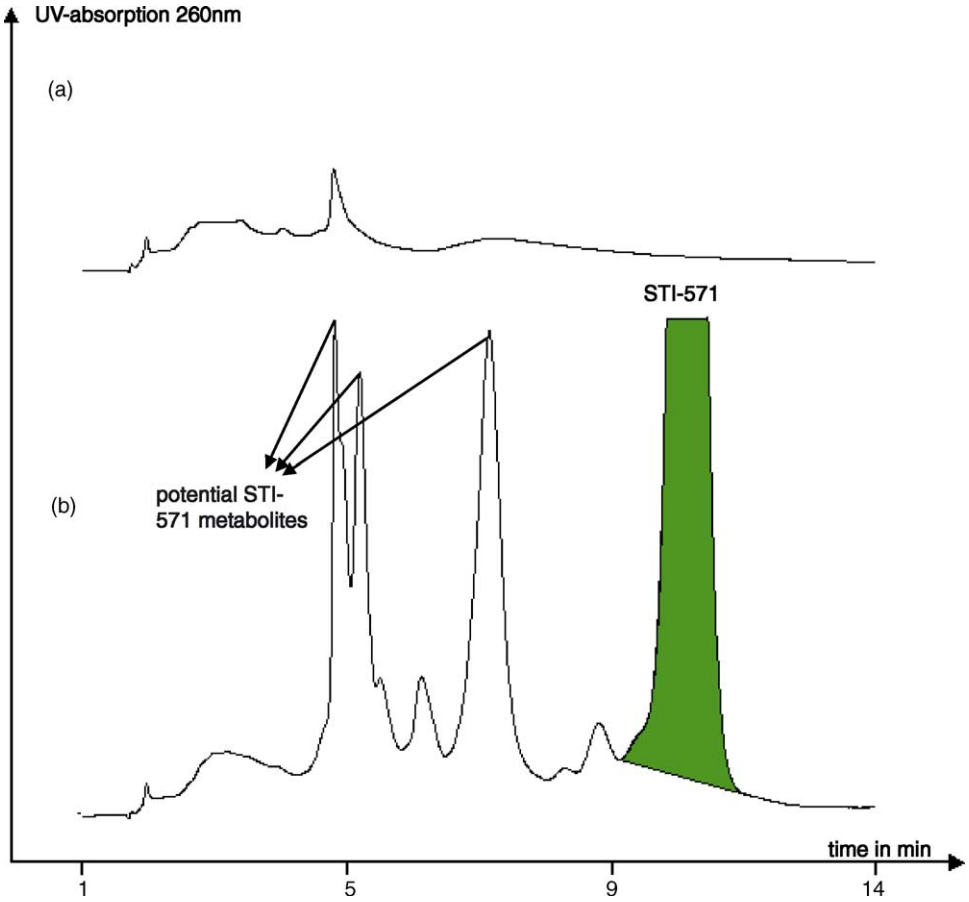

Fig. 7. One to fourteen minutes chromatogram part of (a) blank urine; (b) patient urine under 600 mg/o.d./p.o. STI-571 therapy (blank and patient urine are shown in equal ordinate attenuation).

obviously a "classical" sigmoidal curve describes the cellular STI-571 and *N*-DesM-STI uptake, supporting the theory of a transporter dependent uptake or elimination mechanism. Therefore, the above described procedure of analyzing all involved incubation compartments was again needed to validate the intracellular concentrations in correlation to the extracellular incubation concentrations.

Table 1
Intra-day precision and accuracy in plasma samples

Spiked concentration ($n = 10$ per concentration)	Mean (ng/ml)	S.D. (ng/ml)	CV (% precision)	Accuracy (%)
STI 20 000 ng/ml	20091	520.4	2.6	100.5
N-DesM-STI 20000 ng/ml	20151	433.1	2.2	100.8
STI 8000 ng/ml	7797	310.5	4.0	97.5
N-DesM-STI 8000 ng/ml	7681	291.0	3.8	96.0
STI 4000 ng/ml	3944	176.2	4.5	98.6
N-DesM-STI 4000 ng/ml	4097	228.5	5.6	102.4
STI 1100 ng/ml	1117	19.48	1.7	101.6
N-DesM-STI 1100 ng/ml	1047	28.01	2.7	95.2
STI 600 ng/ml	633.0	14.57	2.3	105.5
N-DesM-STI 600 ng/ml	580.7	8.40	1.5	96.8
STI 110 ng/ml	113.6	4.44	3.9	103.3
N-DesM-STI 110 ng/ml	108.2	6.94	6.4	98.4
STI 60 ng/ml	65.31	3.49	5.3	108.9
N-DesM-STI 60 ng/ml	53.95	4.78	8.9	89.9
STI 25 ng/ml	25.93	2.15	8.3	103.7
N-DesM-STI 25 ng/ml	23.26	0.64	2.8	93.1
STI 10 ng/ml	10.14	0.87	8.6	101.4
N-DesM-STI 10 ng/ml	8.67	0.71	8.2	86.7

Table 2
Intra-day precision and accuracy in urine samples

Spiked concentration ($n = 10$ per concentration)	Mean (ng/ml)	S.D. (ng/ml)	CV (% precision)	Accuracy (%)
STI 20000 ng/ml	20130	521.5	2.6	100.7
N-DesM-STI 20 000 ng/ml	20132	571.9	2.8	100.7
STI 8500 ng/ml	8232	445.9	5.4	96.8
N-DesM-STI 8500 ng/ml	8268	450.8	5.5	97.3
STI 4250 ng/ml	4168	170.5	4.1	98.1
N-DesM-STI 4250 ng/ml	4109	157.0	3.8	96.7
STI 1000 ng/ml	994.6	21.57	2.2	99.5
N-DesM-STI 1000 ng/ml	952.9	12.46	1.3	95.3
STI 400 ng/ml	430.1	18.36	4.3	107.5
N-DesM-STI 400 ng/ml	380.4	18.12	4.8	95.1
STI 70 ng/ml	77.40	4.90	6.3	110.6
N-DesM-STI 70 ng/ml	59.28	5.64	9.5	84.7
STI 25 ng/ml	31.20	2.35	7.5	124.8
N-DesM-STI 25 ng/ml	21.92	1.33	6.1	87.7

Table 3
Intra-day precision and accuracy in RPMI-medium, CSF and cellular samples ($n = 10$ for every experiment)

	Mean (ng/ml)	S.D. (ng/ml)	CV (% precision)	Accuracy (%)
Spiked concentration				
STI-571 110 ng/ml RPMI	121.1	12.49	10.3	110.1
N-DesM-STI 110 ng/ml RPMI	114.2	4.03	3.5	103.8
STI-571 28 ng/ml CSF	30.61	4.85	15.8	109.3
N-DesM-STI 28 ng/ml CSF	24.35	3.23	13.3	87.0
Incubation concentration (6.5 μg/ml in HL-60 cells)				
Cellular STI-571	212676	5295	2.5	31.56[a]
RPMI after incubation	3567	83.14	2.3	52.93[a]
Washing solution	954.8	22.07	23.5	4.18[a]
Reextraction 1	5233	143.5	2.7	4.66[a]
Reextraction 2	811.3	11.50	1.4	0.72[a]
Reextraction 3	119.3	2.52	2.1	0.11[a]
Reextraction 4	15.00	1.00	6.7	0.01[a]
Sum of all compartments (%) for STI-571				94.17[a]
Cellular *N*-DesM-STI-571	225886	5244	2.3	34.19[a]
RPMI after incubation	3467	85.29	2.5	52.48[a]
Washing solution	281.7	30.62	10.9	1.42[a]
Reextraction 1	5806	123.5	2.1	5.27[a]
Reextraction 2	930.7	9.29	1.1	0.85[a]
Reextraction 3	133.0	1.12	0.8	0.12[a]
Reextraction 4	17.33	1.53	8.8	0.02[a]
Sum of all compartments (%) for *N*-DesM-STI				94.34[a]
Incubation concentration (200 ng/ml in HL-60 cells)				
Cellular STI-571	2083	25.42	1.2	10.70[a]
RPMI after incubation	142.33	2.08	1.5	73.12[a]
Washing solution	10.25	1.02	9.9	5.27[a]
Reextraction 1	9.54	0.85	8.9	4.72[a]
Sum of all compartments (%) for STI-571				93.81[a]
Cellular *N*-DesM-STI-571	5301.60	118.9	2.2	28.45[a]
RPMI after incubation	104.00	107.0	103.0	56.17[a]
Washing solution	11.67	1.53	13.1	6.26[a]
Reextraction 1	14.12	0.93	6.6	4.72[a]
Sum of all compartments (%) for *N*-DesM-STI				95.61[a]

[a] Compartment quantity in percentage.

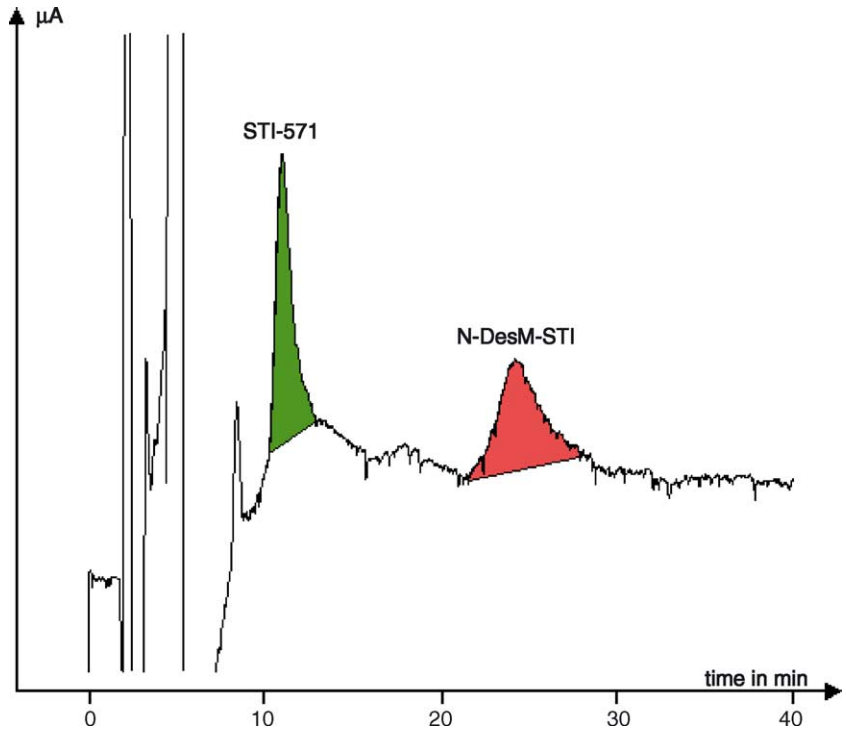

Fig. 8. Spiked plasma with 5 ng/ml STI-571 and *N*-DesM-STI analyzed by EC detection.

Additionally, we approximated the recovery of STI-571 and *N*-DesM-STI extraction from the analyzed cell lines by performing a reextracting procedure four times for the specific cell pellet. Theoretically and in addition to the above described assumptions it was expected that the concentrations after every reextraction will describe a logarithmic decline.

For the determination of accuracy and precision in culture medium measurements, only 10 RPMI samples with 110 ng/ml STI-571 and *N*-DesM-STI were analyzed, since it is highly plausible that the plasma validation results are transferable to RPMI-medium analysis.

4. Results and discussion

4.1. Linearity

Fig. 3 depicts the linearity for STI-571 and *N*-DesM-STI as demonstrated by the mean of 10 analyzed samples for every concentration and the associated correlation curve in plasma and urine obtained over a 3-month validation period.

The linear correlation coefficients were 0.998 and 0.997 for STI-571 and *N*-DesM-STI in plasma while in urine these values were 0.997 and 0.998, respectively. The slope for STI-571 and *N*-DesM-STI in plasma was 0.7158 and 0.7937. The corresponding values for urine were 0.6742 and 0.7053.

4.2. Specifity

No coeluting substance was detected either in the analyzed plasma, urine or CSF samples or in the extracted samples from cell lines and RPMI. After adding STI-571 and *N*-DesM-STI to the respective sample, two new peaks with a mean RT of 9.57 min and 29.98 min were detectable in all sample specimens. The retention time and the UV-spectra from these two peaks were comparable in standard samples, spiked samples and in urine, plasma and CSF samples from patients treated with STI-571 (Figs. 4–6). Moreover, we found three distinct additional peaks only in urine from

Table 4
Pharmacokinetic parameters of STI-571 ($n = 34$) and *N*-DesM-STI ($n = 2$) calculated for 600 mg/o.d./p.o.

Substance	$t_{1/2(\text{terminal})}$ (h)	$t_{1/2(\text{absorption})}$ (h)	AUC (µg h/ml)	C_{max} (µg/ml)	t_{max} (h)	V_{ss} (l)	Clearance $_{total}$ (ml/min)
STI-571	16.7	1.9	71.5	4.8	3.9	151	163.4
CV (%)	25.2	66.1	54.9	73.2	41.8	56.4	37.5
N-DesM-STI	89.7	0.7	10.9	0.47	2.41	1425	187.1
CV (%)	7.7	62.6	20.1	61.9	41.1	32.3	20.4

patients under STI-571 therapy (Fig. 7). These patients had no other medication, no unusual eating habits, or elevated laboratory parameters. We also did not observe these peaks as degradation products in spiked urine. UV-spectra analysis of these peaks shows a quite similar curve with nearly the same maxima and minima compared to STI-571 and *N*-DesM-STI. Taken together, these peaks probably represent the additional metabolites depicted in Fig. 1. We did not validate the measurement of these peaks since it is quite predictable that, due to the chemical structure, these substances have no or only minor biologic activity. Additionally, they represent only a small quantity of STI-571 metabolism in human.

Nevertheless, it seems to be possible to analyze these metabolites on the basis of the described method using a weaker eluent or a gradient system.

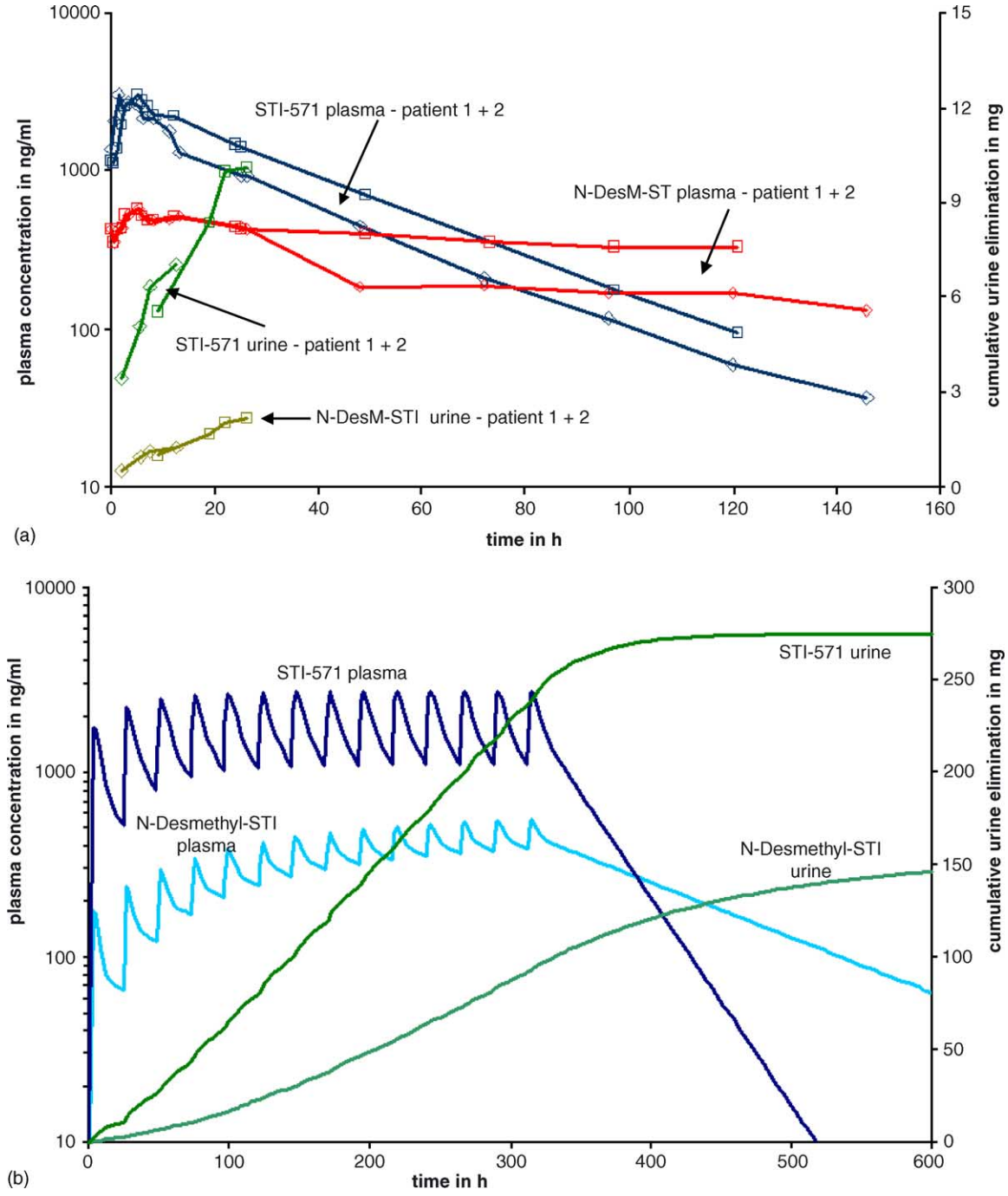

Fig. 9. (a) Concentration–time curves for STI-571 and *N*-DesM-STI after 500 mg/800 mg p.o./o.d. and cumulative urine elimination in steady state: raw data representation; (b) simulated concentration–time curves and cumulative urine elimination for STI-571 and *N*-DesM-STI supposing a 14 day application interval: mean data from two patients and a dose of 600 mg/p.o./o.d. were used for simulation.

4.3. Recovery results in plasma and urine samples

Recovery in plasma samples was 95.1% (range 88.0–112.7%) and 97.5% (range 90.7–101.6%) for STI-571 and *N*-DesM-STI, respectively. This was proven by the mean of 10 aqueous standards compared to 10 plasma samples with a spiked concentration of 2 μg/ml for both substances. Under equal conditions we found a mean recovery of 80.2% (range 77.4–82.5%) for STI-571 and 80.3% (range 76.3–82.9%) for *N*-DesM-STI in urine samples. This slightly different results for plasma and urine can be explained by a small volume contraction in plasma samples using protein precipitation with perchlorid acid in contrast to the native injection of urine samples after 1:1 dilution. Hence, we used two calibration curves for plasma (protein-containing samples) and urine samples (non-protein-containing samples) for a correct calculation of the respective concentrations (Fig. 3).

4.4. Intra- and inter-day results

Tables 1 and 2 depict the intra-day precision and accuracy for plasma and urine samples determined over a validation period of 3 month with 10 analyses according to the respective concentration, respectively. While the correlation coefficient describes the precision for the respective analyzed concentration, the accuracy was calculated by the relation of expected (spiked) concentrations to the mean of the measured concentrations. The results for CSF, RPMI and HL-60 cells are shown in Table 3. For intracellular STI-571

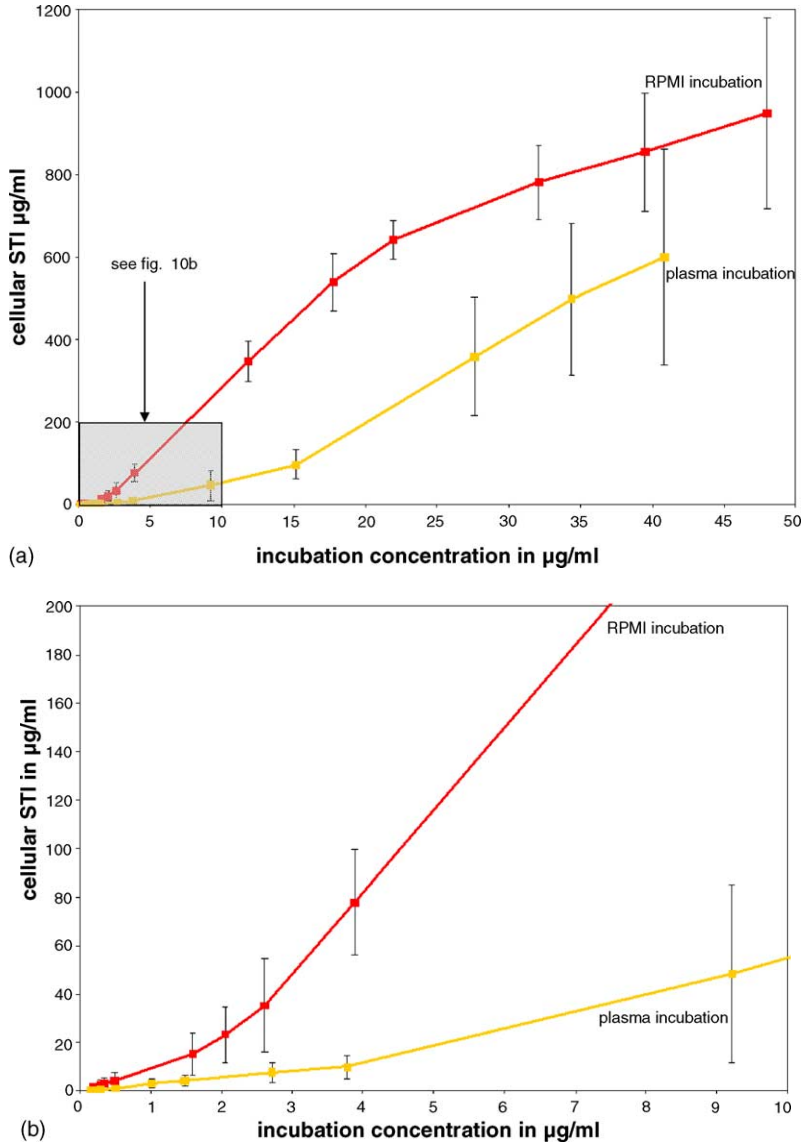

Fig. 10. (a) Cellular STI-571 uptake in 2×10^7 HL-60 cells from STI-571 incubations as depicted over 5 h (the figure shows the data from three independent experiments over a time scale of 3 month: every respective experiment were done in triplicate); (b) detailed representation of (a) for the lower STI-571 concentration range from 0 to 10 μg/ml.

and *N*-DesM-STI the sum of STI-571 and *N*-DesM-STI in all compartments from the incubation experiments is given. This sum was found to be near 100% (96.3%), thus strengthening the assumption that the "true" intracellular concentration was measured. Moreover, the reextraction experiments done in triplicate show that with the first sample preparation about 85% of intracellular STI-571 and *N*-DesM-STI were extracted from the cell pellet and that the following extractions lead to a logarithmical decline as shown in Table 3. Furthermore, in this setting we analyzed the rediffusion from intracellular STI-571 to the washing solution and found that up to 10% of the intracellular compounds was redistributed within 5 min to the medium (data not shown). Based on this observation the centrifugation time for the washing step in all experiments described above was 5 min.

The inter-day precision determined in plasma samples (22 experiments, spiked concentration 1 μg/ml STI-571 and *N*-DesM-STI, validation period 7 month) was 8.31 and 6.88%, for STI-571 and *N*-DesM-STI, while for the accuracy we found a value of 100.25 and 99.59%, respectively. In urine samples (12 experiments, spiked concentration 750 ng/ml STI-571 and *N*-DesM-STI, validation period 7 month) these value were 16.45% (STI-571) and 14.83% (*N*-DesM-STI) for the precision and 97.35% (STI-571) and 100.34% (*N*-DesM-STI) for the accuracy.

Using the alternative sample preparation consisting of liquid/liquid extraction and electrochemical detection as described above we found a precision of 12.3% and an accuracy of 87.25% by processing 10 plasma samples with 5 ng/ml (Fig. 8). This demonstrates that EC detection and limits of detection <10 ng/ml are thereby possible, but the higher effort and the complete sufficient limit of detection reached by UV-detection makes the usefulness of this method questionable.

4.5. Assay applications

The assay was used for the measurements of STI-571 and *N*-DesM-STI in various sample specimens (Figs. 9–11). We were able to show that relevant plasma concentrations of STI-571 and *N*-DesM-STI after discontinuing therapy were detectable for more than 10 days (Fig. 9b). This observation may have clinical relevance since unexpected toxicity may occur when conditioning therapy is performed shortly after discontinuation of STI-571 in patients with CML. Moreover, we found that *N*-DesM-STI, which was separated from patient urine has nearly the same biological activity as STI-571 (data not shown). An additional pharmacokinetic result is the very low concentration of STI-571 and *N*-DesM-STI in the CSF. The concentrations measured for both STI-571 and *N*-DesM-STI were approximately only 1–3% of those detected in plasma samples even when the patient suffered from a meningeosis leukemica (data not shown).

Table 4 depicts the pharmacokinetic parameters calculated from a total of 34 patients analyzed with the described method. A complete description of this data will be reported separately.

The determination of intracellular concentrations of STI-571 in HL-60 cells demonstrated a rapid influx of the drug when compared to the extracellular concentrations as illustrated by the hyperbolic incline of the cellular uptake both in human plasma and RPMI (Figs. 10 and 11). These results lead to the conclusion that apparently small alterations of extracellular concentrations of STI-571 may result in a drastic shift of the intracellular concentration. This phenomenon is most significant at extracellular concentrations between 2 and 20 μg/ml leading to the assumption that an active transport mechanism may be involved in the intracellular uptake of STI-571. Moreover, the lower intracellular concentration of STI-571 after incubation in human

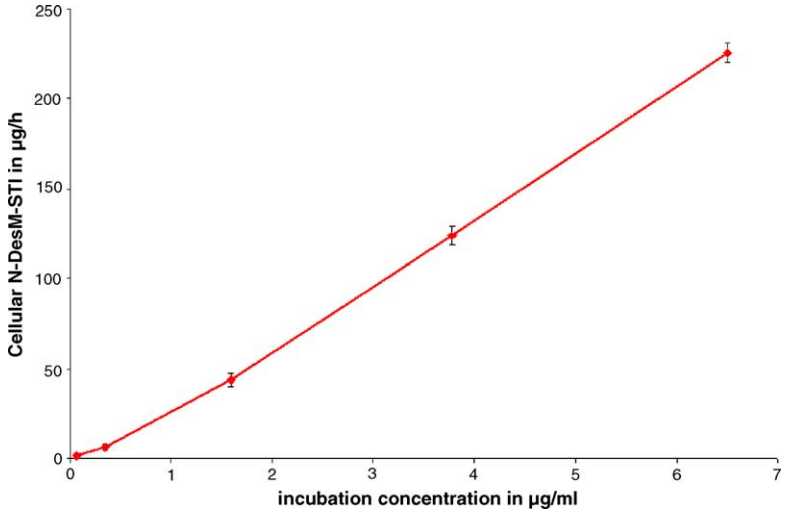

Fig. 11. Cellular *N*-DesM-STI uptake in 2×10^7 HL-60 cells from *N*-DesM-STI incubations as depicted over 5 h in RPMI-medium, analysis were done in triplicate.

plasma demonstrate that the influx is dependent on the free proportion of STI. The opportunity to obtain validated intracellular measurements of STI-571 and *N*-DesM-STI, described for the first time here, was used by our group to investigate the influence of *p*-glycoprotein on STI-571 and *N*-DesM-STI uptake [28], the role of protein binding for cellular uptake and to investigate pharmacokinetic interactions with Ara-C and other chemotherapeutic agents. The detailed results of this studies will also be reported separately.

5. Conclusions

The method described here allows for the determination of STI-571 and *N*-DesM-STI in plasma, urine, CSF, culture-media and in the intracellular compartment with common HPLC equipment without the need of cost intensive liquid chromatography–tandem mass spectrometry. It can be the platform for a even more sensitive HPLC method by using electrochemical detection, but so far the actual limit of detection (10 ng/ml in plasma and 25 ng/ml in urine) fulfill all demands needed for pharmacokinetic studies. The described HPLC exhibits a broad linearity from 10 ng/ml to 20 μg/ml and a fast and easy sample preparation. Using an autosampler in combination with an electric valve-triggering-switch, available in almost all current HPLC equipment, a semi-automated application is possible allowing for routine measurements.

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