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Article

Development and validation of liquid chromatography-tandem mass spectrometry method to quantify dasatinib in plasma and its application to a pharmacokinetic study

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Dasatinib, a potent oral multi-targeted kinase inhibitor against Src and Bcr-Abl, can decrease inflammatory response in sepsis. A simple and cost-effective method for determination of an effective dose dasatinib was established. This method was validated in human plasma, with the aim of reducing the number of animals used, thus, avoiding ethical problems. Dasatinib and internal standard lopinavir were extracted from 180 uL of plasma using liquid-liquid extraction with methyl tert-butil ether, followed by liquid chromatography coupled to triple quadrupole mass spectrometry in multiple reaction monitoring mode. For the pharmacokinetic study, 1 mg/kg of dasatinib was administered to mice with and without sepsis. The method was linear over the concentration range of 1-98 ng/mL for DAS in mice and human plasma, with r^2>0.99 and presented intra- and interday precision within the range of 2.3 – 6.2 and 4.3 – 7.0%, respectively. Further intra- and interday accuracy was within the range of 88.2 – 105.8 and 90.6 – 101.7%, respectively. The mice with sepsis showed AUC0-t = 2076.06 h*ng/mL and Cmax = 102.73 ng/mL and mice without sepsis presented AUC0-t = 2128.46 h*ng/mL. Cmax = 164.5 ng/mL. The described analytical method was successfully employed in pharmacokinetic study of DAS in mice.

Keywords: LC-MS/MS. Liquid-liquid extraction. Dasatinib. Mice plasma. Pharmacokinetics.

INTRODUCTION

Dasatinib (DAS), a Src- family tyrosine kinase (SFK) inhibitor acts on both Abl- and Src-family tyrosine kinases (Weisberg *et al.*, 2007), is currently used in patients with chronic myeloid leukemia (LMC), acute lymphoblastic leukemia with Philadelphia positive chromosome (LLA Ph+) and in lymphoblastic LMC (Lowe *et al.*, 2014; Rabenau *et al.*, 2014). SFK plays critical roles in inflammatory disease models (Berton, Mócsai and Lowell, 2005; Kovács *et al.*, 2014), including cell adhesion, and migration through the vessel wall (Mócsai, Walzog and Lowell, 2015). Besides its effect on malignant cells, dasatinib also inhibits leukocytes critical functions for the inflammatory response (Futosi *et al.*, 2012). For example, it decreases systemic TNF production followed by lipopolysaccharide (LPS) injection in Src and Btk dependent fashion (Fraser *et al.*, 2009).

Sepsis is a medical condition initiated by severe infection with exacerbated systemic inflammation (Munford, 2006) followed by immunosuppression resulting in multiple organ failure and high morbidity and mortality (Gaieski *et al.*, 2013). According to recent Centers for Disease Control and Prevention (2016) estimates, 1.7

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million adult Americans become septic each year; of those, nearly 270,000 Americans die, and one in three patients who die in a hospital have sepsis (National Center for Emerging and Zoonotic Infectious Diseases, 2016).

In sepsis, the detection of microbes by immune cells is followed by activation of intracellular pathways including the Src pathway. Src kinase suppression in mice (Src knock-out mice) showed a decrease in inflammatory response challenged by toxic molecules and reperfusion injury (McPherson *et al.*, 2009). Pharmacological SFK inhibitors protected animals from sepsis or viral infections (Okutani *et al.*, 2006).

In this context, we performed a pharmacokinetic study in control and septic mice to evaluate dasatinib plasma concentration. As our model was abdominal sepsis, the cecum ligation and perforation (CLP) model, our aim was to ensure that dasatinib would reach blood stream in a concentration high enough to inhibit SFK (Gonçalves-de-Albuquerque *et al.*, 2018).

Liquid chromatography coupled to triple quadrupole mass spectrometry (LC-MS/MS) has been demonstrated to be a powerful technique for the quantitative determination of drugs and metabolites in biological fluids. Because of its high sensitivity and selectivity, LC-MS/MS method has been chosen to quantify SFK inhibitores in mice plasma (Kamath *et al.*, 2008; Hsieh *et al.*, 2009; Patel *et al.*, 2014; Wen *et al.*, 2015; Cao *et al.*, 2015).

Herein we used CLP model to induce sepsis and describe the development and validation of a simple and fast LC-MS/MS method to determine DAS in mice plasma of both control and septic animals. This was the first method that used liquid-liquid extraction (LLE) in the preparation of mice plasma, furthermore to the validation being performed in human plasma, reducing the number of experimental animals used, without compromising the quality of the analytical method.

MATERIAL AND METHODS

Chemicals

DAS (free base, >99%) was purchased from LC laboratories, Woburn, Massachusetts, USA. Lopinavir (LOP) was donated by Abbott. Blank plasma was obtained from Instituto Estadual de Hematologia Arthur Siqueira Cavalcanti – Hemorio, Rio de Janeiro, Brazil. Methanol (MeOH), dimethyl sulfoxide (DMSO) and methyl tert-butil ether (MTBE), HPLC-grade and formic acid and ammonium acetate, analytical grade were purchased from Tedia Brazil (Rio de Janeiro, RJ, Brazil). Ultra-pure water (H2O) was obtained from an Elga USFilter system (Garden Grove, California, USA).

Instrumentation and Chomatographic Conditions

HPLC system (1200 series, Agilent Technologies, Germany) is connected with API 3200 triple quadrupole mass spectrometer (SCIEX, Toronto, Canada) using multiple reaction monitoring (MRM). A turbo electrospray interface (ESI) in positive ionization mode was used. The determination of optimal potential settings and MS/MS transitions were established by DAS and IS infusion into the MS/MS detector at a concentration of 500 ng/mL in 50:50 v/v of solution H2O:MeOH. The parameters of the ion source were optimized to the following settings: an ionization spray voltage of 4500V, curtain gas of 30 psi, heater gas of 60 psi, turbo ion spray gas of 40 psi, a source temperature of 550 ºC and entrance potential of 5V. High-purity nitrogen was used as the collision gas. Quantitation was performed using MRM of the transitions at *m/z* 488.2 > 401.3 (DAS) and *m/z* 629.4 > 155.2 (LOP). Data processing was performed on Analyst 1.5.1 software (SCIEX, Toronto, Canada). The chromatography was performed on ACE 5 AQ (4.6 x 150mm, 5um) column (Advanced Chromatography Technologies, USA) at 40º C temperature. The mobile phase used consisted of ammonium acetate buffer 5 mmol/L and MeOH (15:85 v/v), each with 0.1% formic. The flow rate was 0.8 mL/min.

Preparation of Calibration Standards and Quality Control Samples

Standard stock solution of DAS (1 mg/mL) was prepared in DMSO. DAS dilution (10 ug/mL) was made from DAS standard stock solution with diluent (MeOH). This solution was added to drug-free human plasma

to obtain DAS calibration standards of 1, 3, 6, 12, 24, 48 and 96 ng/mL. Quality control (QC) samples were also prepared as a bulk on an independent weighing of standard drug at concentrations of 1 (lower limit of quantitation - LLOQ), 2 (low quality control - LQC), 36 (middle quality control - MQC), 72 (high quality control - HQC) and 192 ng/mL (dilution quality control - DQC) from stock solutions of DAS. The calibrations standards and quality control samples were stored in the freezer at below -20º C until analysis.

Sample preparation

A plasma sample aliquot (180 µL) was mixed with a 20 µL of IS solution (LOP at 500 ng/mL) and vortexed briefly. The resulting samples were subjected to liquid-liquid extraction with 2 mL of MTBE. The mixture was centrifuged at 18 ˚C for 20 min at 3000 rpm. The organic layer was removed, transferred to a clean glass tube and dried in a vacuum sample concentrator (CentriVap®) for 30 min, without heating. The dried residue was reconstituted in 180 µL mixture of 85% MeOH and 15% 0.05M ammonium acetate by vortexing for 1 min. Finally, a 20 µL of each sample was transferred to an auto sampler vials and injected into a LC-MS/MS.

Calibration Curves

The analytical curves of DAS were constructed in the concentrations ranging from 1 to 96 ng/mL in human plasma. The calibration curve was made by using an instrument response (ratio of DAS peak area to IS peak area) against the DAS concentration (ng/mL) for 3 consecutive days by weighted $1/x^2$ quadratic regression model. The fitness of the calibration curve was confirmed by back-calculating the concentrations of calibration standards.

Bionalytical method validation

The method was validated in accordance with the Brazilian Health Surveillance Agency (ANVISA), RDC n° 27/2012 (Agência Nacional de Vigilância Sanitária, 2012).

During the analytical validation, a test of comparison between the human plasma and the naïve, sham and CLP mice plasma was performed. Three injections of blank plasma of each species were performed and the chromatograms obtained were compared. The aim of this test was to ensure that blank plasmas of different animal groups (naïve, sham and CLP) are similar, so that we could replace the mice plasma with human plasma in the assay validation.

Selectivity

The selectivity of the method was determined by blank human plasma samples from six different sources (4 normal, 1 hyperlipidemic and 1 hemolyzed samples) to test the potential interferences of endogenous compounds coeluted with DAS and IS. The chromatographic peaks of DAS and IS were identified on the basis of their retention times and MRM responses. The mean peak area of LLOQ for DAS and IS at corresponding retention time in blank samples should not be more than 20 and 5%, respectively.

Carry over

The carry over was evaluated by three injections of the same blank sample, one before and two immediately after the injection of a processed HQC sample. The results were compared with those obtained from processed LLOQ samples, and should not be greater than 20% of the response of the LLOQ sample and 5% of the response of the IS.

Matrix Effect

The matrix effect due to the plasma matrix was used to evaluate the ion suppression/enhancement in a signal comparing the absolute response of QC samples in solution with that of reconstitution samples extracted from the blank plasma samples spiked with the analyte.

For evaluating the matrix effect, 8 samples of different sources (4 normal, 2 hyperlipidaemic and 2 hemolyzed samples), processed and subsequently added to the analyte in the concentrations of LQC and HQC

and IS, and solution in the same concentrations were analyzed. For each sample, the IS-normalized matrix factor (NMF) that is expressed as (matrix analyte response / matrix IS response) / (solution analyte response / solution IS response) was obtained. The coefficient of variation of NMF for all samples should not be greater than 15 %.

Linearity

The human plasma with the addition of 1, 3, 6, 12, 24, 48 and 96 ng/mL of DAS in triplicates was used for the construction of calibration curves. Correlation coefficients (r^2) were obtained by using quadratic regression model in the whole range of tested concentrations.

Accuracy and precision

Replicate analysis (n=7) of quality control samples at the five concentrations (LLOQ, LQC, MQC, HQC and DQC) was used for the intra-assay precision and accuracy determination. Interassay accuracy and precision were determined by repeated analysis performed on three different days. The concentration in each sample was determined using calibration standards prepared on the same day. Precision was calculated as the coefficient of variation (CV %) within a single run (intra-assay) and between different assays (inter-assays), and accuracy was calculated as percentage of deviation between nominal and measured concentration.

During the routine analysis of mice samples, duplicate control samples at three concentration levels (LQC, MQC, HQC) were assayed. The analytical series were considered valid and accepted only if the percentage of deviation (bias) between theoretical and back-calculated (experimental) concentrations for each calibration level and quality control samples were less than ±15%, and less than 20% at the limit of quantification.

Stability

The stability of DAS in biological matrix at different storage conditions was evaluated and the results were expressed as percentage recoveries (concentration of sample under different storage condition/concentration sample freshly-prepared). Precision was calculated as the coefficient of variation (CV %) and accuracy as percentage of deviation between nominal and measured concentration.

Plasma spiked with DAS in the concentrations of LQC and HQC (n=5) was kept at room temperature for 6 h, after being processed and injected (short-term stability). Freeze/thaw stability was determined after freezing (-20 °C) and thawing QC samples for three cycles. Stability of the processed sample was also measured after the sample processing and its stay on the injector for 19 h.

Application of the LC-MS/MS method

The pharmacokinetic analysis of DAS in mice followed the methodology described for (Gonçalves-de-Albuquerque *et al.*, 2018). Briefly, male Swiss Webster (SW) mice (25-30 g) at least 8 weeks old and healthy looking were obtained from the Oswaldo Cruz Foundation breeding unit, Rio de Janeiro, Brazil. The animals received DAS (1 mg/kg) by gavage in a volume of 100 µL per animal 30 min before and 6 h after the induction of sepsis. During sepsis, the animals were divided into two groups, CLP (cecal ligation and puncture) and Sham (control animals that received the same volume replacement and antibiotics treatment administered to animals with CLP). Blood samples were collected at 0.25, 0.5, 1, 2, 4, 8.16 and 23.75h after DAS second administration. The samples were immediately centrifuged at 5.000 rpm for 10 min, the plasmas were transferred to other tubes and stored at -20 ºC until analysis. The procedures described were approved by the Oswaldo Cruz Institute Committee under license number CEUA/IOC L-015/2015. Pharmacokinetic parameters, including area under the concentration-time curve (AUC), maximum plasma concentration (C_{max}), time to reach to maximum concentration (T_{max}) were estimated by a non-compartmental analysis using Phoenix WinNonlin (Pharsight Inc., USA, version 8.1).

RESULTS AND DISCUSSION

Method Development

Mass spectrometric conditions were optimized for getting highly sensitive and selective analysis. Initially, MS/MS conditions were optimized by syringe infusion of standard solutions of DAS and LOP (IS). MRM technique with positive ESI was chosen for the assay development. $[M + H]^+$ was the predominant ion in the Q1 spectrum. The Q1 for DAS and IS was 488.2 and 629.4, respectively, and were used as precursor ion to obtain product ion spectra. The most sensitive mass transitions were from m/z 401.3 for DAS and m/z 155.2 for the IS. The selected m/z transitions, declustering potential (DP), collision energy (CE), and collision cell exit potential (CEP) for analyte and PI are reported in Table I.

TABLE I - Optimized mass spectrometry parameters for dasatinib and lopinavir as internal standard

	MRM transition	DP (V)	CE (V)	CEP (V)
DAS	488.2 → 401.3	86	33	6
LOP	629.4→155.2	41	55	4

DAS, dasatinib; LOP, lopinavir; MRM, multiple reaction monitoring; DP, declustering potential; CE, collision energy, CEP, collision cell exit potential.

Mass spectrometry parameters, fragmentation pattern, and mode of ionization are the main task in mass spectrometry tuning to obtain respective fragmented ions and response for both DAS and IS which are shown in Figure 1.

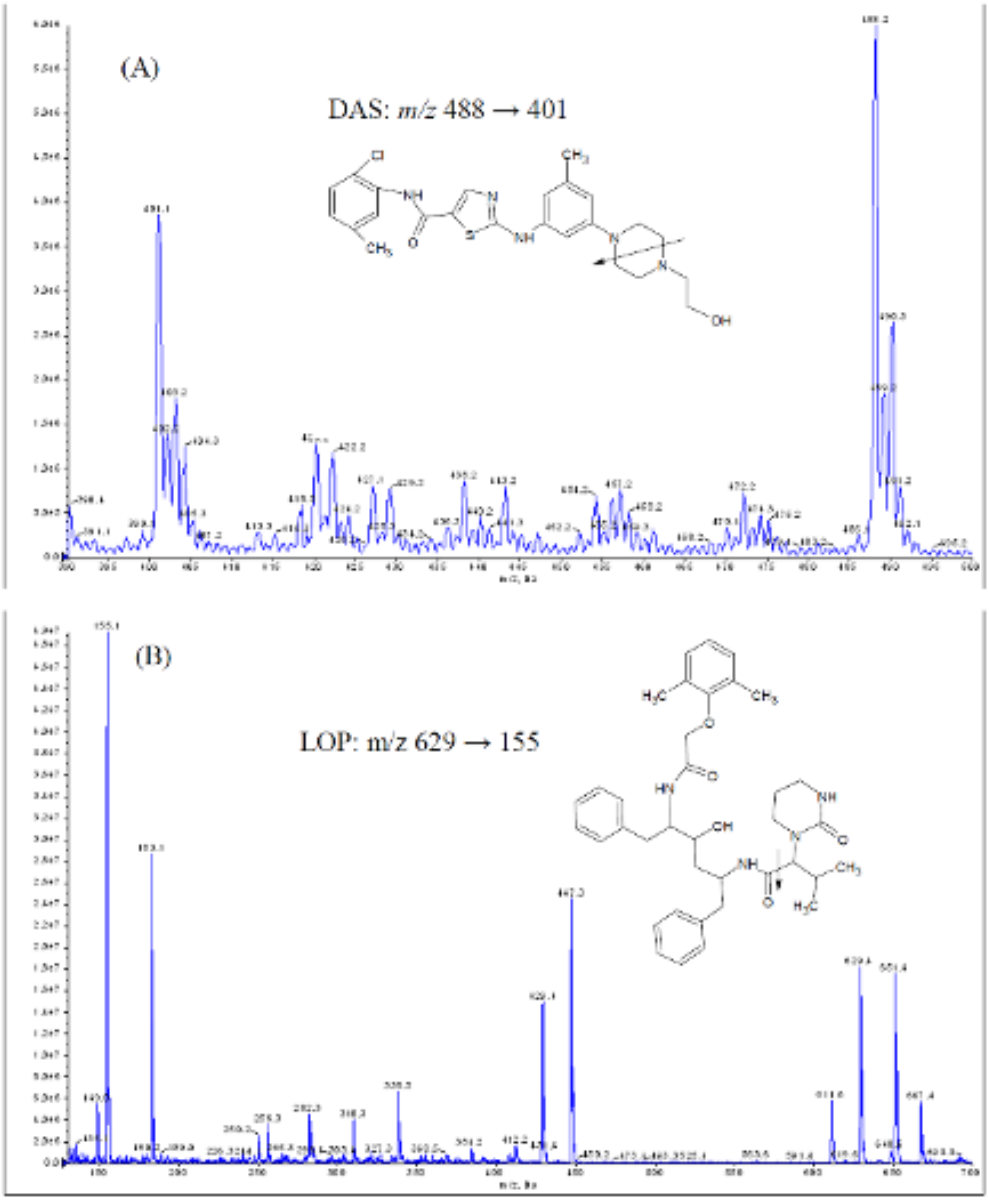

FIGURE 1 - Representative product ion mass spectra of (a) DAS and (b) the internal standard LOP.

The chromatographic conditions were optimized in order to obtain good resolution and symmetric peak shapes for the DAS and IS. We tested several columns, composition of mobile phase and flow-rate of mobile phase and found that the best mobile phase consisted of ammonium acetate buffer 5mmol/L and MeOH (15:85 v/v), each with 0.1% formic. The addition of acid modifiers (0.1% formic acid and 5 mmol/L ammonium formate) to the mobile phase increases the protonation of DAS and thus enhances sensitivity. Moreover, the high proportion of organic solvent eluted the DAS at retention time 3.06 min at a flow rate of 0.8 mL/min, produced good peak shapes and permitted a total run time of 5 min.

One of the most critical parameters in the development of bioanalytical methods is sample preparation, which involves selective isolation of analyte of interest from the matrix, minimization or elimination of matrix components in the extracted samples, and, if necessary, enrichment of analyte to ensure assay sensitivity. The most commonly employed sample preparation methods in LC-MS/MS bioanalysis generally include protein precipitation (PPT), liquid-liquid extraction (LLE), and solid phase extraction (SPE) (Li, Jian, Fu, 2019).

Several analytical methods for detection and quantification of DAS using LC-MS/MS have been published. Most of these methods use extraction by PPT for plasma sample cleanup (Kamath *et al.*, 2008; Hsieh *et al.,* 2009; Patel *et al.*, 2014; Wen *et al.*, 2015; Cao *et al.*, 2015; Haouala *et al.*, 2009; Mičová *et al.*, 2012; Lankheet *et al.*, 2013; Andriamanana *et al.*, 2013; Van Erp *et al.*, 2013; Huynh *et al.*, 2017). Three methods use SPE (Maher *et al.*, 2018; Bouchet *et al.*, 2011; Furlong *et al.*, 2012), while four methods use LLE: a study in cells using methyl tert-butyl ether (MTBE):acetonitrile (3:1, v/v):1M ammonium formate pH 3.5 (8:1, v/v) mixture (Roche *et al.*, 2009) and three methods in human plasma using hexane:ethyl acetate (30:70, v/v) (Kralj *et al.*, 2012), butyl acetate:butanol (4:1, v/v) (Birch *et al.*, 2013) and ethyl acetate added to the sodium hydroxide solution (Zeng *et al.*, 2017).

Our method of sample preparation consists in LLE in two steps: extraction with MTBE and evaporation of the organic phase with samples concentrator, which allowed us to obtain a limit of quantification of 1 ng/mL. The LLE is a simple sample preparation technique that involves the extraction of the analyte of interest or unwanted interference components from one liquid phase (e.g. biological samples) to another immiscible liquid phase (e.g. organic solvent), resulting in sample clean-up (Li, Jian, Fu, 2019). MTBE was found as the best solvent for extraction because, besides evaporating rapidly, it produces clean extracts without the matrix effect, and a consistent recovery for the analyte.

Bionalytical method validation

To perform the bioanalytical method validation, a large volume of mice plasma is required. Studies have shown that animal plasma can be replaced with human plasma without compromising the selectivity, precision and accuracy of the analytical method (Jacobson *et al.*, 2011).

In this study, to avoid ethical problems with the indiscriminate use of animals, a comparison assay was carried out between the human plasma and the naïve, sham and CLP mice plasma.

Figure 2 shows that there was no difference between the total ion chromatograms of the blank plasma injections and, therefore, during the validation and calibration curves for quantification of DAS, we used human plasma, obtained from a blood bank.

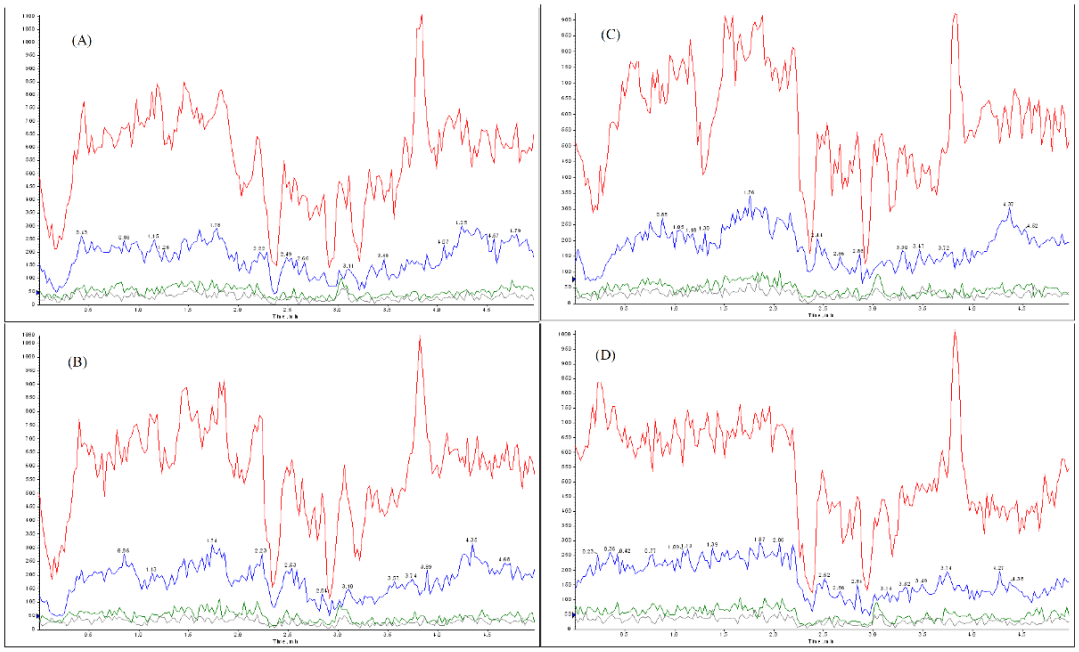

FIGURE 2 - Total ion chromatogram (m/z 488.2 > 401.3, m/z: 488.2 >232.2, and m/z 629.4 > 155.2) of a blank human plasma (A) in comparison with total ion chromatogram of naïve (B), sham (C) and CLP (D) blank mice plasma, in the same conditions of HPLC-MS/MS.

Selectivity

No peaks from endogenous compounds were observed at the drugs retention time in any of the 6 evaluated blank plasma extracts. The responses in blank plasma were always below 14.9% of the signal at the LLOQ of 1ng/mL for DAS and below 0.62% for IS. Figure 3 shows a representative ion chromatogram for the blank plasma (a) and LLOQ (b) with 20 µL injection volume.

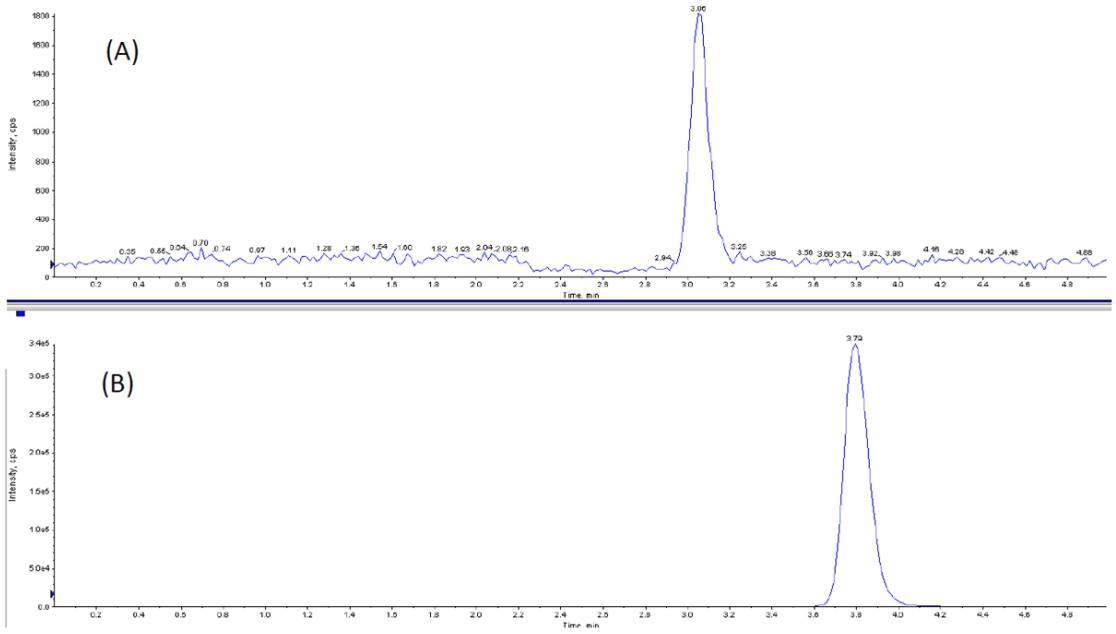

FIGURE 3 - Representative ion chromatogram for the blank plasma (A) and LLOQ plasma (B) with 20 µL injection volume.

Carry over

The carry over was evaluated by means of three injections of the same blank sample as follows: one before and two immediately after the injection of a sample processed HQC. The peak of DAS and IS corresponded to 6.83% and 0.53% of the respective peaks in the sample of LLOQ. Carry-over was therefore considered acceptable.

Matrix Effect

The mean normalized matrix factor for DAS was 0.71 with CV values ≤ 14%, which indicates that the method is free from any major ion suppression or enhancement in the MRM channel used for the quantification of DAS.

Linearity

The seven-point calibration curve was linear over the concentration range of 1-96 ng/mL. The calibration model was selected based on the data by quadratic regression with $1/x^2$ weighting factor. Over the considered concentration range, the regression coefficient r^2 of the calibration curves was always greater than 0.99 with backcalculated calibration samples within ± 15% (± 20% at LLOQ).

Accuracy and precision

Accuracy and precision determined with LLOQ, LQC, MQC, HQC and DQC samples are given in Table II. For the intra-assay experiments, precision and accuracy ranged from 2.3 to 6.2% and from 88.2 to 105.8%. Further, in between different assays (inter-assays), precision and accuracy ranged from 4.3 to 7.0% and from 90.6 to 101.7%, respectively.

TABLE II - Precision and accuracy (analysis with spiked plasma samples at five different concentrations) determined by repeated analysis performed on three different days (inter-assays) and within the same day (intra-assay)

		LLOQ	LQC	MQC	HQC	DQC
Nominal concentration (ng/mL)		1.00	2.00	36.00	72.00	192.00
Intra-assay (n=7)						
1	mean ± sd. (ng/mL)	0.88 ± 0.05	1.80 ± 0.04	36.57 ± 1.07	70.47 ± 2.00	194.49 ± 3.88
	accuracy (%)	88.24	90.00	101.59	97.88	101.29
	precision (%CV)	6.20	2.39	2.94	2.84	5.98
2	mean ± sd. (ng/mL)	0.93 ± 0.03	1.96 ± 0.08	38.07 ± 0.88	74.31 ± 2.29	177.75 ± 2.49
	accuracy (%)	92.57	97.79	105.75	103.21	92.58
	precision (%CV)	2.90	4.34	2.30	3.09	4.20
3	mean ± sd. (ng/mL)	0.91 ± 0.02	1.85 ± 0.05	35.16 ± 1.21	69.43 ± 2.59	173.40 ± 2,15
	accuracy (%)	91.11	92.57	97.66	96.43	90.31
	precision (%CV)	2.40	2.46	3.45	3.73	3.71
Inter-assay (n=21)						
	mean ± sd. (ng/mL)	0.91 ± 0.04	1.88 ± 0.09	36.60 ± 1.58	71.40 ± 3.07	182.54 ± 4.26
	accuracy (%)	90.64	93.82	101.67	99.17	95.07
	precision (%CV)	4.41	4.71	4.32	4.30	7.00

LLOQ, lower limit of quantitation; LQC, low quality control; MQC, middle quality control; HQC, high quality control; DQC, dilution quality control.

Stability

Quantification of the DAS in plasma that was subjected to 3 freeze-thaw cycles (-20◦C to room temperature) showed the stability of the analyte. The concentrations ranged from 92.00 to 109.00% for DAS. No significant degradation was observed even after a 19-hour storage period in the autosampler tray, and the final concentrations of DAS were found between 88.90 and 100.00%. The room temperature stability of DAS in QC samples after 6 h was also evaluated. The concentrations were ranged between 86.40 and 108.00% for DAS. These results confirmed the stability of DAS, as shown in Table III.

TABLE III - Stability of dasatinib in human plasma samples

Spiked plasma concentration (ng/mL n=5)	Mean concentration measured (ng/mL)	Precision (%CV)	Accuracy (%)
Room temperature stability for 6 hr in plasma			
2.00	1.93 ± 0.11	5.95	96.30
72.00	75.94 ± 1.95	2.57	105.47
Three freeze-thaw cycles			
2.00	1.88 ± 0.04	5.65	94.40
72.00	79.30 ± 3.15	1.24	96.17
Auto sampler stability for 19 hr			
2.00	1.89 ± 0.11	2.09	93.80
72.00	69.24 ± 0.86	3.97	110.14

Pharmacokinetic study

The validated method has been successfully applied to a pharmacokinetic study performed in male SW mice, in which the plasma concentration was determined for up to 24 h after administration of 1mg/kg of DAS. The plasma concentration time profiles of Dasatinib for CLP and Sham animals are shown in Figure 4. The main pharmacokinetic parameters in the mice that underwent CLP were as follows: AUC_{0-t} = 2076.06 h*ng/mL; C_{max} = 102.73 ng/mL; T_{max} = 16h; C_{min} = 60.78 ng/mL, T_{mix} = 4h. For the Sham they were AUC_{0-t} = 2128.46 h*ng/mL; C_{max} = 164.5 ng/mL; T_{max} = 1h; C_{min} = 72.83 ng/mL, T_{mix} = 8h. Although the septic animals showed C_{max} and AUC_{0-t} values lower than those of the Sham animals, the C_{min} was over 14.9 ng/mL, which is the concentration able to inhibit 90% of phosphorylation of pBCR-ABL protein. Thus, it was observed that the administration of 1mg/kg DAS in mice with sepsis was sufficient to keep DAS at pharmacological levels up to 24h after administration and inhibit SFK.

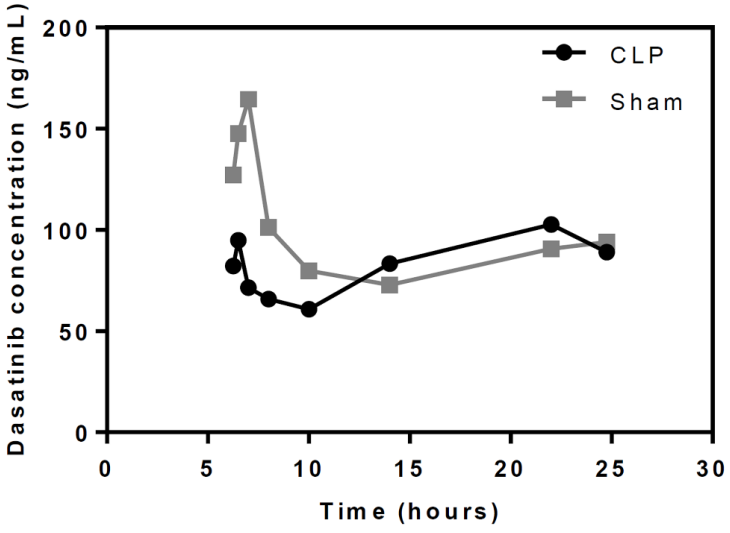

FIGURE 4 - Mean plasma concentration vs. time after administration of dasatinib in Swiss Webster mice. Dasatinib was given orally 30 min before and 6 after sepsis after cecal ligation and puncture (CLP). Sham-treated animals were used as control.

By combining LLE and LC/MS-MS, our method was developed and validated since it provides accuracy, precision and great sensitivity, which is of particular importance for drugs such as dasatinib that was found in low concentrations in mice plasma (Luo *et al.*, 2006). Our methodology was validated in human plasma, wich made it cheaper, besides avoiding the ethical problems resulting from the excessive use of animals. Furthermore, it is suitable to be used in clinical studies due to its validation in human plasma, its high sensitivity and its good reproducibility using a small volume of plasma.

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DECLARATIONS OF INTEREST

None

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