LC-ESI-MS/MS Determination of GSK-199, A Novel Reversible PAD4 Inhibitor in Mice Plasma and its Application to a Pharmacokinetic Study in Mice

Gurulingappa Hallur¹, Buchi Reddy Purra¹, Jeyaraj Duraismwamy A¹, Suresh Sulochana P¹, Pavan Kumar Nagasuri VSP², Rudresh Gangaiah², Mohd Zainuddin², SriRam Rajagopal¹ and Ramesh Mullangi²

¹Medicinal Chemistry Department, Jubilant Biosys Limited, Industrial Suburb, Yeshwanthpur, Bangalore, Karnataka, India
²Drug Metabolism and Pharmacokinetics, Jubilant Biosys Limited, Industrial Suburb, Yeshwanthpur, Bangalore, Karnataka, India

Abstract

A simple, specific, sensitive and rapid LC-ESI-MS/MS method has been developed and validated for the quantification of GSK-199 in mice plasma using warfarin as an internal standard (IS). Sample preparation was accomplished through a liquid-liquid extraction process. Chromatographic separation of GSK-199 and IS was performed on a Chromolith column using an isocratic mobile phase comprising 0.2% formic acid in water and acetonitrile (25:75, v/v) at a flow rate of 1.0 ml/min. Elution of GSK-199 and IS occurred at ~1.32 and 1.81 min, respectively. The total chromatographic run time was 2.50 min. A linear response function was established in the concentration range of 2.52-1009 ng/ml. Method validation was performed as per regulatory guidelines and the results met the acceptance criteria. The intra- and inter-day accuracy and precision were in the range of 1.75-12.3% and 4.99-12.3%, respectively. GSK-199 was found to be stable under various stability conditions. This novel method has been applied to a pharmacokinetic study in mice.

Keywords: GSK-199; LC-MS/MS; Method validation; Mice plasma; Pharmacokinetics

Introduction

Protein arginine deiminase (PAD) is an enzyme that plays an important role in gene expression, turning on genetic code into functional products in the body. It regulates various processes such as apoptosis, innate immunity, neutrophil extracellular trap (NET) formation and pluripotency etc. Protein arginine deiminases (PADs1-4 and PAD6) catalyzes citrullination of arginine residues, which affects numerous physiological and pathological processes [1]. PAD4 plays an important role in human diseases and hence has been the prime focus among all the PADs. Dysregulated PAD4 activity has been observed in a number of diseases, specifically in inflammation and human cancers. In rheumatoid arthritis PAD4 is expressed in macrophages and neutrophils of synovial joint and presence of PAD4 accelerates NETosis in rheumatoid arthritis patients [2]. GSK-199 (Figure 1), chemically (R)-3-(aminopiperidin-1-yl) (2-(1-ethyl-1H-pyrrolo[2,3-b]pyridin-2-yl)-7-methoxy-1-methyl-1H-benzo[d]imidazol-5-yl)methanone is a novel, selective and reversible small-molecule inhibitor of PAD4 and NET production [3]. It acts by novel mechanism of action and crystallographic studies confirmed its selectivity and activity by identifying its key conformational changes at enzyme's active site. Recent study showed that PAD4 inhibition is sufficient enough to show efficacy in rheumatoid arthritis in mice and corroborates that PAD4 as a promising therapeutic target to address the unmet medical need to show efficacy in rheumatoid arthritis in mice and corroborates that PAD4 inhibition is sufficient enough to show efficacy in rheumatoid arthritis in mice and corroborates that PAD4 inhibition is sufficient enough to show efficacy in rheumatoid arthritis in mice and corroborates that PAD4 inhibition is sufficient enough to show efficacy in rheumatoid arthritis in mice and corroborates that PAD4 inhibition is sufficient enough to show efficacy in rheumatoid arthritis in mice and corroborates that PAD4 inhibition is sufficient enough to show efficacy in rheumatoid arthritis in mice.

To date there is no LC-MS/MS method reported for quantification of GSK-199 in any biological matrix. In this paper, we report the development and validation of a simple, specific, sensitive and reproducible LC-MS/MS method for quantitation of GSK-199 in mice plasma. The method was successfully applied to quantitate levels of GSK-199 in mice pharmacokinetic studies.

Materials and Methods

GSK-199 (purity ≥ 98%) was purchased from Cayman Chemicals, Michigan, USA. Warfarin (purity >99%) was purchased from Sigma-Aldrich, St. Louis, USA. HPLC grade acetonitrile and methanol were purchased from J.T. Baker, Centre Valley, PA, USA. Analytical grade formic acid was purchased from S.D Fine Chemicals, Mumbai, India. All other chemicals and reagents were of analytical grade and used without further purification. The control BalbC mice K<sub>E</sub>-EDTA plasma sample was procured from Animal House, Jubilant Biosys, Bangalore.

Instrumentation and chromatographic conditions

A Shimadzu HT (Shimadzu, Japan) LC system equipped with degasser (DGU-20A5), binary pump (LC-20AD) along with auto-sampler (SIL-HTC) was used to inject 10 µl aliquots of the processed samples on a Chromolith Performance RP-18e column (100 × 4.6

Figure 1: Structural representation of GSK-199.
mm), which was maintained at 40 ± 1°C. The solvents used for chromatography were filtered through a 0.45 μm membrane filter (X55225050) (Millipore, USA or equivalent) and then degassed ultrasonically for 5 min. An isotropic mobile phase comprising 0.2% formic acid in water and acetonitrile (25:75, v/v) was delivered at a flow rate of 1.0 ml/min.

Quantitation was achieved by MS/MS detection in positive ion mode for analyte and IS using a MDS Sciex (Foster City, CA, USA) API-4000 mass spectrometer, equipped with a TurboVapnSpray interface at 600°C temperature and 5500 V ion spray voltage. The source parameters viz., curtain gas, GS1, GS2 and CAD were set at 35, 45, 55 and 10 psi. The compound parameters viz., declustering potential (DP), entrance potential (EP), collision energy (CE) and collision cell exit potential (CXP) 30, 10, 33, and 5 V for GSK-199 and 67, 10, 25, and 8 V for IS. Detection of the ions was performed in the multiple reaction monitoring (MRM) mode, monitoring the transition of the m/z 433 precursor ion to the m/z 333 product ion for GSK-199 and m/z 309 to 163 for IS. Quadrupole Q1 and Q3 were set on unit resolution. The dwell time was 150 msec. The analytical data were processed by Analyst software (version 1.6.3).

**Standard solutions**

GSK-199 and IS were weighed accurately into volumetric flasks using an analytical micro balance. The primary stock solutions of GSK-199 and IS were prepared at 202 and 1000 μg/ml, respectively in DMSO. The primary stock solutions of GSK-199 and IS were stored at -20°C, which were found to be stable for twenty days (data not shown). The primary stock of analyte was successively diluted in Milli-Q water:methanol (2.8, v/v) to prepare secondary stocks and working solutions to prepare calibration curve (CC) for GSK-199 was prepared in Milli-Q water:acetonitrile (1:1, v/v). Working stock solutions were stored approximately at 4°C for a week. Working stocks were used to prepare plasma calibration standards. A working IS solution (100 μg/ml) was prepared in DMSO:methanol (1:9, v/v). Blank mice plasma was screened prior to spiking to ensure that it was free from endogenous interference at retention times of GSK-199 and IS. Eight-point calibration standards samples (2.52-1009 ng/ml) were prepared by spiking the blank mice plasma with appropriate concentration of GSK-199. Samples for the determination of precision and accuracy were prepared by spiking control mice plasma in bulk with GSK-199 at appropriate concentrations 2.52 ng/ml (LLOQ, lower limit of quantitation), 7.57 ng/ml (MQC, low quality control), 454 ng/ml (MQC, medium quality control) and 807 ng/ml (HQC, high quality control) and 50 μl plasma aliquots were distributed into different tubes. All the samples were stored at -80 ± 10°C.

**Sample preparation**

To an aliquot of 50 μl plasma 10 μl of IS (100 μg/ml) solution and 1.0 ml of ethyl acetate were added and vortex mixed for 5 min; followed by centrifugation for 5 min at 14,000 rpm in a refrigerated centrifuge (Eppendorf 5424R) maintained at 5°C. The organic layer (900 μl) was separated and evaporated to dryness at 40°C using a gentle stream of nitrogen (Turbovap®, Zymark®, Kopkinton, MA, USA). The residue was reconstituted in 200 μl of the mobile phase and 10 μl was injected onto LC-MS/MS system for analysis.

**Bioanalytical method validation**

A full validation according to the US FDA guidelines [5] was performed for GSK-199 in mice plasma. The method was validated with respect to specificity, selectivity, carryover, linearity, accuracy, precision, percentage recovery, matrix effects, stability, dilution integrity and incurred samples reanalysis. Method selectivity was evaluated by analyzing six different K2.EDTA plasma lots including one each of lipemic and haemolyzed plasma (i.e., without analyte and the IS), zero samples (i.e., blank plasma with the IS) and LLOQ samples were used to confirm the absence of potential endogenous interfering peaks in chromatograms. The LLOQ was determined as the concentration that has a precision of <20% of the relative standard deviation and accuracy between 80 and 120% of the theoretical value. Effect of carryover in the succeeding runs were also evaluated by injecting blank plasma sample into LLOQ sample → blank plasma sample → ULOQ sample → blank plasma sample. For linearity establishment, a total of four batches of calibration curves were analyzed to validate the method. Six replicates of LLOQ QC, LQC, MQC and HQC samples were analyzed along with a calibration curve for intra-day precision and accuracy results, whereas for inter-day accuracy and precision were assessed by analyzing four batches of samples on three consecutive days. The precision (%) at each concentration level from the nominal concentration should not be greater than 15%, except for LLOQ QC where it should be 20%. The accuracy (%) must be within ±15% of their nominal value at each QC level except LLOQ QC where it must be within ± 20%. The recovery of GSK-199 determined at LQC (7.75 ng/ml), MQC (454 ng/ml) and HQC (807 ng/ml), whereas for IS the concentration of 100 μg/ml. Recovery for the analyte and the IS was calculated by comparing the mean peak response of pre-extraction spiked samples (spiked before extraction; n=6) to that of non-extracted samples (neat samples; n=6) at each QC level. Matrix effects for GSK-199 and the IS were assessed by comparing the mean peak areas at LQC and HQC concentration after extracting into blank plasma with the mean peak areas for neat analyte solutions in the mobile phase. Plasma samples stability at room temperature (6 h), after repeated freeze-thaw cycles (3 cycles) in auto-sampler (for 24 h) and long-term for 30 days (at -80 ± 10°C) were conducted at both LQC and HQC levels. These stability samples were processed and quantified against freshly spiked calibration curve standards along with freshly spiked QC samples. Samples were considered to be stable if assay values were within the acceptable limits of accuracy (± 15% SD) and precision (≤ 15% RSD). Upper concentration limit of the GSK-199 can be extended by performing the dilution integrity experiment. Six replicates each at a concentration of about 5 times of the ULOQ were diluted 5- and 10-fold with screened blank plasma. Incurred sample reanalysis (ISR) was also performed.

**Pharmacokinetic study**

All the animal experiments were approved by Institutional Animal Ethical Committee (IAEC/JDC/2017/121). Male Balb/C mice (n=24) were procured from Vivo Biotech, Hyderabad, India. The animals were housed in Jubilant Biosys animal house facility in a temperature (22 ± 2°C) and humidity (30-70%) controlled room (15 air changes/hour) with a 12:12 h light:dark cycles, had free access to rodent feed (Altromin Spezialfutter GmbH & Co. KG., Im Seelenkamp 20, D-32791, Lage, Germany) and water for one week before using for experimental purpose. Following ~4 h fast (during the fasting period animals had free access to water) animals were divided into two groups (n=12/group). Group I animals (21-25 g) received GSK-199 orally at 10 mg/kg (suspension formulation comprising 0.1% Tween-80 and 0.5% of methyl cellulose; strength: 1.0 mg/ml; dose volume: 10 ml/kg), whereas Group II animals (25-30 g) received GS0-199 intravenously [5% DMSO, 5% Solubol:absolute alcohol (1:1, v/v) and 90% of normal saline; strength: 0.1 mg/ml; dose volume: 10 ml/kg] at 2.0 mg/kg dose. Post-dosing serial blood samples (100 μl, sparse sampling was done and at each time point three mice were used for blood sampling) were
collected using Micropipettes (Microcaps®; catalogue number: 1-000-0500) through tail vein into polypropylene tubes containing K$_2$EDTA solution as an anti-coagulant at 0.25, 0.5, 1, 2, 4, 8, 10 and 24 h (for oral study) and 0.12, 0.25, 0.5, 1, 2, 4, 8 and 24 h (for intravenous study). Plasma was harvested by centrifuging the blood using Biofuge (Hereaus, Germany) at 1760 g for 5 min and stored frozen at -80 ± 10°C until analysis. Animals were allowed to access feed 2 h post-dosing.

The criteria for acceptance of the analytical runs encompassed the following:

(i) 67% of the QC samples accuracy must be within 85-115% of the nominal concentration,

(ii) Not less than 50% at each QC concentration level must meet the acceptance criteria [5].

Plasma concentration-time data of GSK-199 was analyzed by non-compartmental method using Phoenix WinNonlin (Version 7.0).

Results

Mass spectroscopy

In order to optimize the most sensitive ionization mode for GSK-199 and IS, electro-spray ionization (ESI) full scans were carried out both in positive and negative ion detection modes and found abundant [M+H]$^+$ ions in 433 and 309 for GSK-199 and IS, respectively. Following detailed optimization of mass spectrometry conditions, MRM reaction pair of m/z 433 precursor ion to the m/z 333 was used for quantification for GSK-199. Similarly, for IS MRM reaction pair of m/z 309 precursor ion to the m/z 163 was used for quantification purpose. The fragmentation pattern of GSK-199 is shown in Figure 2.

Liquid chromatography

Initial feasibility experiments of various mixture(s) of solvents such as acetonitrile and methanol using different buffers such as ammonium acetate, ammonium formate and formic acid along with altered flow-rates (in the range of 0.8-1.4 ml/min) were performed to optimize for an effective chromatographic resolution of GSK-199 and IS (data not shown). A variety of analytical columns (Inertsil, Atlantis, Kromasil, Hypersil, Chomolith etc.) were employed to obtain good and reproducible response with short run time. The resolution of peaks was best achieved with an isocratic mobile phase comprising 0.2% formic acid:acetonitrile (25:75, v/v) at a flow rate of 1.0 ml/min. Chromolith Performance RP-18e column (100 × 4.6 mm) was found to be suitable with sharp and symmetric peak shapes among few other columns tested in the method optimization process (data not shown). GSK-199 and IS eluted at ~1.32 and 1.81 min, respectively.

Optimization of sample preparation and recovery

The mean percent recovery of GSK-199 was at LQC, MQC and HQC was found to be 59.7 ± 7.66, 63.5 ± 6.68 and 63.8 ± 7.65%, respectively. The recovery of IS was 101 ± 5.31%. Liquid-liquid extraction with ethyl acetate found to be simple and efficient sample clean up devoid of matrix effect and interference from endogenous plasma components (Figure 3).

Matrix effect

Mean absolute matrix effect for GSK-199 in control mice plasma was 92.3 ± 2.55 and 96.3 ± 2.53% at LQC and HQC, respectively. The matrix effect for IS was 101 ± 2.03%. These results indicate the absence of matrix effects that can obscure the quantification of GSK-199 and IS.

Specificity and selectivity

Figures 4a-4c show chromatograms for the blank mice plasma (free of analyte and IS; Figure 4a), blank mice plasma spiked with GSK-199 at LLOQ and IS (Figure 4b) and an in vivo plasma sample obtained at 0.25 h after oral administration of GSK-199 along with IS (Figure 4c). The retention time of GSK-199 and IS was ~1.32 and 1.81 min, respectively. The total chromatographic run time was 2.5 min. Analysis of blank mice plasma from six different sources showed no interferences at the retention times of GSK-199 and IS confirming the selectivity of the method. Sample carryover effects were not observed owing to the use of 80% methanol in Milli-Q water as needle washing solution.

Calibration curve

The plasma calibration curve was constructed in the linear range using eight calibration standards viz., 2.52, 5.04, 10.1, 25.2, 252, 504, 757 and 1009 ng/ml. The calibration standard curve had a reliable

![Figure 2: Mass fragmentation pattern of GSK-199.](image)

![Figure 3: Overlay chromatograms showing the matrix effect for (a) GSK-199 (b) IS.](image)
Figure 4: Typical MRM chromatograms of GSK-199 (left panel) and IS (right panel) in (a) mice blank plasma (b) mice blank plasma spiked with GSK-199 at LLOQ (2.52 ng/ml) and IS (c) a 0.25 h in vivo plasma sample showing GSK-199 peak obtained following oral administration to mice along with IS.
reproducibility over the standard concentrations across the calibration range. The average regression ($n=4$) was found to be ≥ 0.996 for GSK-199. The lowest concentration with the RSD <20% was taken as LLOQ and was found to be 2.52 ng/ml. The accuracy observed for the mean of back-calculated concentrations for four calibration curves for GSK-199 was within 90.3-110%; while the precision (CV) values ranged from 0.85-11.1%.

**Accuracy and precision**

Accuracy and precision data for intra- and inter-day plasma samples for GSK-199 are presented in Table 1. The assay values on both the occasions (intra- and inter-day) were found to be within the accepted variable limits.

**Stability**

The predicted concentrations for GSK-199 at 7.57 and 807 ng/ml samples deviated within ± 15% of the fresh sample concentrations in a battery of stability tests viz., bench-top (6 h), in-injector (24 h), repeated three freeze/thaw cycles and freezer stability at -80 ± 10°C for at least for 30 days (Table 2). The results were found to be within the assay variability limits during the entire process.

**Dilution effect**

Standard curve can be extended up to 5044 ng/ml without affecting the final concentrations. The precision (% CV) values for dilution integrity were between 2.38 and 4.66 for both (5- and 10-fold) the dilutions.

**Incurred samples reanalysis**

All the 12 samples selected for ISR met the acceptance criteria. The back calculated accuracy values ranged between 88.9 to 101% from the initial assay results.

**Pharmacokinetic study**

The sensitivity and specificity of the assay were found to be sufficient for accurately characterizing the plasma pharmacokinetics of GSK-199 in BalbC mice. Profiles of the mean plasma concentration versus time for oral and intravenous studies were shown in Figure 5. GSK-199 was quantifiable up to 10 and 8 h following oral and intravenous administration, respectively. In the present study following intravenous administration the clearance (Cl) and volume of distribution (Vd) were found to be 25.0 ml/min/kg and 3.40 l/kg, respectively. Following oral administration maximum plasma concentrations (C max: 1073 ng/ml) attained 1.0 h (Tmax). The AUC0-∞ (area under the plasma concentration-time curve from time zero to infinity) was found to be 4192 and 1342 ng*h/ml, by oral and intravenous routes, respectively. The terminal half-life (t½) was 1.58 and 1.48 h by intravenous and oral route, respectively. The absolute oral bioavailability was 63.6%.

<table>
<thead>
<tr>
<th>Theoretical concentration (ng/ml)</th>
<th>Run</th>
<th>Measured concentration (ng/ml)</th>
<th>Mean</th>
<th>SD</th>
<th>RSD</th>
<th>Accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Intraday variation (Six replicates at each concentration)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.52</td>
<td>1</td>
<td>2.89</td>
<td>0.28</td>
<td>9.54</td>
<td>114</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2.85</td>
<td>0.30</td>
<td>10.7</td>
<td>113</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2.53</td>
<td>0.21</td>
<td>8.32</td>
<td>112</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>2.25</td>
<td>0.08</td>
<td>3.48</td>
<td>89.4</td>
<td></td>
</tr>
<tr>
<td>7.57</td>
<td>1</td>
<td>7.28</td>
<td>0.13</td>
<td>1.75</td>
<td>96.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>7.00</td>
<td>0.45</td>
<td>6.37</td>
<td>92.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>7.06</td>
<td>0.50</td>
<td>7.11</td>
<td>93.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>6.87</td>
<td>0.18</td>
<td>2.66</td>
<td>90.7</td>
<td></td>
</tr>
<tr>
<td>454</td>
<td>1</td>
<td>477</td>
<td>49.3</td>
<td>10.3</td>
<td>105</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>479</td>
<td>33.9</td>
<td>7.07</td>
<td>98.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>450</td>
<td>55.4</td>
<td>12.3</td>
<td>99.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>475</td>
<td>51.5</td>
<td>10.9</td>
<td>105</td>
<td></td>
</tr>
<tr>
<td>807</td>
<td>1</td>
<td>843</td>
<td>84.8</td>
<td>10.1</td>
<td>104</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>853</td>
<td>94.4</td>
<td>11.1</td>
<td>106</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>886</td>
<td>90.3</td>
<td>10.2</td>
<td>110</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>895</td>
<td>82.5</td>
<td>9.22</td>
<td>111</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Inter day variation (Twenty four replicates at each concentration)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.52</td>
<td></td>
<td>2.75</td>
<td>0.34</td>
<td>12.3</td>
<td>112</td>
<td></td>
</tr>
<tr>
<td>7.57</td>
<td></td>
<td>7.05</td>
<td>0.35</td>
<td>4.99</td>
<td>93.2</td>
<td></td>
</tr>
<tr>
<td>454</td>
<td></td>
<td>463</td>
<td>52.3</td>
<td>11.3</td>
<td>102</td>
<td></td>
</tr>
<tr>
<td>807</td>
<td></td>
<td>897</td>
<td>101</td>
<td>11.3</td>
<td>108</td>
<td></td>
</tr>
</tbody>
</table>

where; RSD: Relative standard deviation (SD $\times$ 100/Mean)

**Table 1:** Intra- and inter-day precision and accuracy determination of GSK-199 quality controls in mice plasma.
In this paper, we report the method development and validation of a bioanalytical method for quantification of GSK-199 in mice plasma. Critical evaluation and optimization of buffer, mobile phase composition, flow-rate and analytical column are very important to obtain good resolution of peaks of interest from the endogenous components, which in turn affect sensitivity and reproducibility of the method. We have optimized the sample extraction process mainly to achieve high extraction recovery with negligible or low matrix effects in order to improve sensitivity and reliability of LC-MS/MS analysis. The attained LLOQ (2.52 ng/ml) was sufficient to quantify GSK-199 to characterize the pharmacokinetics in mice. Due to non-availability of deuterated GSK-199 to use it as an IS, several commercial drugs were evaluated to find out a suitable IS. Finally, warfarin was found to be the best for present purpose based on the chromatographic elution, ionization and reproducible and good extraction efficiency. The acceptable limit for both intra- and inter-day accuracy and precision is ± 20%. In this method, both intra- and inter-day accuracy and precision are well within this limit, indicating that the developed method is precise and accurate for GSK-199.

In summary, a method using LC-ESI-MS/MS for the determination of GSK-199 in mice plasma employing simple liquid-liquid extraction was developed. The method is simple, specific and sensitive. Additionally, demonstrates good accuracy and precision and is fully validated according to FDA guidelines. The method showed suitability for pharmacokinetic studies in mice.

**Discussion**

The therapies available for rheumatoid arthritis have evolved from salicylates, to non-steroidal anti-inflammatory drugs, corticosteroids, and disease-modifying anti-rheumatic drugs. Despite the fact that the above therapeutic interventions are effective in achieving remission, these are less tolerable, do not result in drug-free remission, and many patients show persistent disease activity even during treatment. Evidences linking PAD4 activity to various diseases suggest that PAD4-specific inhibitors possess clinical utility for the treatment of rheumatoid arthritis and other diseases. F-amidine and Cl-amidine are first set of compounds discovered, which inhibits PAD4 irreversibly [6]. Though F- and Cl-amidine are known to be potent PAD4 inhibitors, their role in inhibiting PAD4 involved in NET formation remains poorly understood. Both compounds lack of selectivity among the PADs and do not have desirable pharmacokinetic properties. Hence, new classes of reversible PAD4 inhibitors have been discovered. GS199 is the reversible PAD4 inhibitor, which is currently being used extensively as a tool compound to understand the molecular mechanism of PAD4 in rheumatoid arthritis. Preclinical pharmacokinetics has great important influence on the development and investigation of potential candidates with better advice for the further drug design. Pharmacokinetic studies can both provide toxicological and clinical information and direct optimization of drug candidates; as a result, they play necessary parts in drug discovery and development.

To date there is no published validated method available for the quantification of any PAD4 inhibitor in any of the biological matrices.

In summary, a method using LC-ESI-MS/MS for the determination of GSK-199 in mice plasma employing simple liquid-liquid extraction was developed. The method is simple, specific and sensitive. Additionally, demonstrates good accuracy and precision and is fully validated according to FDA guidelines. The method showed suitability for pharmacokinetic studies in mice.

**References**
