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A novel LC-ESI-MS/MS assay method for the determination of anagrelide in human plasma by using a solid phase extraction technique and its application to a pharmacokinetic study

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A simple, rapid and sensitive liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) assay method has been developed and validated for the determination of anagrelide in human plasma samples using nevirapine as internal standard (IS). The analyte and the IS were extracted from 100 μL of human plasma using solid-phase extraction with no drying, evaporation and reconstitution steps. The chromatographic separation was achieved on a C_{18} column by using a mixture of methanol and 0.1% formic acid in 5 mM ammonium acetate (80 : 20, v/v) as the mobile phase at a flow rate of 1.0 mL min^{-1}. The linearity of the method was established in the concentration range 0.05–10.0 ng mL^{-1} with $r^2 \geq 0.99$. Method validation was performed as per FDA and EMEA guidelines and the results met the acceptance criteria. Data acquisition was done by multiple reaction monitoring (MRM) on a triple quadrupole mass spectrometer in the positive ionization mode. The intra-day and inter-day precision (%CV) and accuracy results in five validation batches across five concentration levels were well within the acceptance limits. The validated method was successfully applied to a pharmacokinetic study in healthy South Indian male subjects under fasting conditions with 0.5 mg anagrelide capsules.

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Introduction

Essential thrombocythaemia or essential thrombocytosis (ET) is a rare chronic blood disorder characterized by a sustained elevation in platelet count and megakaryocyte hyperplasia by megakaryocytes in the bone marrow.[1] Anagrelide is an orally active quinazolin used in the treatment of ET.[2–4] It has been shown to reduce the platelet count over a dose of 1 to 4 mg per day and thereby reduce the incidence of thrombohaemorrhagic symptoms for 1 to more than 28 months. The drug is metabolized by cytochrome P450 (CYP) 1A2.[3,5] The mechanism by which anagrelide reduces the blood platelet count is still under investigation. The drug is well tolerated and can be administered orally.

As per the available literature few analytical methods based on RP-HPLC have been reported for the determination of anagrelide in pharmaceutical preparations[6–8] and in biological samples.[4] Similarly, one GC-MS method[9] and two LC-MS based methods[3,10] have been reported in the literature for analysis of anagrelide in plasma samples. The reported GC-MS method has employed liquid–liquid extraction followed by derivatization for sample preparation, which is a complicated and labor-intensive procedure.

Zhu *et al.*, (2005)[10] reported a LC-MS method for the plasma concentration range of 0.05–7.5 ng mL^{-1} using 0.50 mL plasma sample volume. This method employs liquid–liquid (L–L) extraction, evaporation, drying and reconstitution for sample preparation. Moreover, the method utilizes 0.5 mL plasma sample volume which may not be favorable for routine subject analysis. In another report, cardiovascular safety, effects of caffeine and food intake on pharmacokinetics of anagrelide were investigated in healthy subjects.[3]

This paper presents the development and validation of a simple, selective and sensitive liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) assay method for the determination of anagrelide in human plasma using nevirapine as an internal standard (IS). The method employs a simple solid-phase extraction (SPE) technique for sample preparation and has a sensitivity equal to that reported by Zhu *et al.* (2005) using a smaller plasma volume (100 μL). SPE is the most popular sample preparation technique due to following advantages: high recovery, effective sample preparation, requires less volume of organic solvent compared

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to LLE, ease of operation and greater possibility of automation.[11–13] This method ensured the estimation of anagrelide in real-time samples collected from healthy male subjects with desired accuracy and precision to support a pharmacokinetic study in healthy volunteers. Furthermore, this is the first report on successful demonstration of assay reproducibility through incurred sample reanalysis (ISR) for anagrelide.

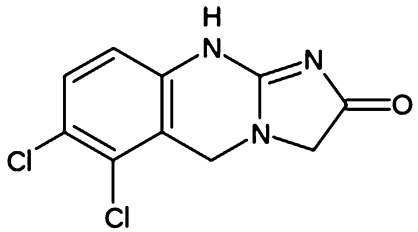
Experimental

Standards and reagents

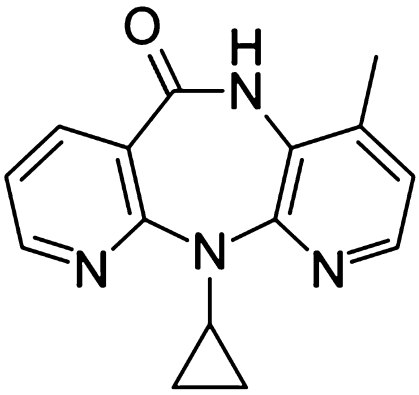
The reference standard sample of anagrelide (>95.00%) was obtained from Clearsynth Labs Limited (Mumbai, India), while nevirapine (>99.35%) was from Hetero Labs Limited (Hyderabad, India). The representative chemical structures are presented in Fig. 1. Water used for the LC-MS/MS analysis was prepared by using a Milli Q water purification system procured from Millipore (Bangalore, India). HPLC grade methanol was purchased from J.T. Baker (Phillipsburg, USA), while analytical grade formic acid and ammonium acetate were from Merck Ltd (Mumbai, India). The control K2 human plasma sample was procured from Deccan's Pathological Labs (Hyderabad, India).

LC-MS/MS instrument and conditions

An HPLC system (Shimadzu, Kyoto, Japan) equipped with a Discovery® HS C_{18} column (50 mm × 4.6 mm, 5 μm) (Make: Supelco), a binary LC-20AD prominence pump, an auto sampler (SIL-HTc) and a solvent degasser (DGU-20A_3) was used for the study. Aliquots of 20 μL of the processed samples were injected into the column, which was kept at 40 ± 2 °C. An isocratic mobile phase composed of a mixture of methanol and 0.1% formic acid in 5 mM ammonium acetate (80 : 20, v/v) was used to separate the analyte from endogenous components and pumped at a flow rate of 1.0 mL min^{-1} into the electrospray ionization chamber of the mass spectrometer. Quantification was achieved with MS-MS detection in positive ion mode for the analyte and the IS using an AB Sciex API-4000 mass spectrometer (Foster City, CA, USA) equipped with a Turboionspray ™ interface at 500 °C. The ion spray voltage was set at 5500 V. The source parameters *viz.* the nebulizer gas (GS1), auxiliary gas (GS2), curtain gas and collision gas were set at 35, 35, 20, and 8 psi, respectively. The compound parameters *viz.* the declustering potential (DP), collision energy (CE), entrance potential (EP) and collision cell exit potential (CXP) were 75, 40, 10, and 11 V for anagrelide and 43, 33, 10, and 10 V for the IS. Detection of the ions was carried out in the multiple-reaction monitoring mode (MRM), by monitoring the transition pairs of *m*/*z* 256.0 precursor ion to the *m*/*z* 199.1 ion for anagrelide and *m*/*z* 267.40 precursor ion to the *m*/*z* 226.20 product ion for the IS. Quadrupoles (Q1 and Q3) were set on unit resolution. Date acquisition was performed with Analyst Software™ (version 1.4.2).



Anagrelide



Nevirapine (IS)

Fig. 1 Chemical structures of anagrelide and nevirapine (IS).

Preparation of stock and working solutions

Two standard stock solutions of anagrelide were prepared separately in HPLC grade methanol (1 mg mL^{-1}). Their concentrations were corrected according to the actual amount weighed accounting for its potency. Working standard solutions necessary for plotting the calibration curve (CC) samples were prepared by appropriate dilution of one of the above stock solutions of anagrelide using a mixture of methanol and water (50 : 50, v/v; diluent). Quality control (QC) samples for determination of accuracy and precision were prepared by appropriate dilution of the second standard stock solution prepared above using the same diluent. The concentrations of the QC samples are selected from five different levels of the calibration curve range.

A 1 mg mL^{-1} of nevirapine stock solution was prepared by dissolving the compound in HPLC grade methanol. The working concentration of nevirapine (1500 ng mL^{-1}) was achieved from the above stock solution using the diluent.

Preparation of calibration curve standards and quality control samples in human plasma

Six lots of K2 EDTA human plasma were screened and used to prepare calibration curve standards, quality control samples and dilution integrity (DIQC) samples. After bulk spiking, aliquots of 200 μL for CCs and 200 μL for QCs of spiked plasma samples were pipetted out into prelabelled micro-centrifuge tubes (2 mL) and then all the bulk spiked samples were stored in a deep freezer at −70 ± 10 °C.

Calibration samples were prepared by spiking 950 μL of control K2 EDTA human plasma with 50 μL working standard

solution of the analyte in bulk, to obtain anagrelide concentration levels of 0.05, 0.10, 0.25, 0.50, 0.99, 2.00, 3.99, 6.00, 8.01 and 10.0 ng mL^{-1} as a single batch at each concentration. Similarly, quality control (QC) samples were also prepared in bulk based on independent weighing of the standard drug, at concentrations of 0.05 (lower limit of quantitation, LLOQ), 0.15 (low quality control, LQC), 1.22 (medium quality control, MQC1), 5.08 (MQC2) and 9.07 ng mL^{-1} (high quality control, HQC) as a single batch at each concentration.

Sample preparation protocol

All the frozen subject samples, calibration standards and quality control samples were thawed and equilibrated at room temperature prior to analysis. A 100 μL aliquot of the human plasma sample was mixed with 20 μL of the internal standard working solution (1500 ng mL^{-1} of nevirapine). The sample mixture was loaded onto a Strata-X 33 μm polymeric sorbent cartridge (30 mg/1 mL) that was pre-conditioned with 1.0 mL of methanol followed by 1.0 mL of water. The extraction cartridge was washed with 1.0 mL of 0.2% formic acid solution followed by 1.0 mL of 5% methanol. The analyte and IS were eluted with 0.5 mL of mobile phase. An aliquot of 20 μL of the extract was injected into the chromatographic system.

Method validation procedures

Thorough and complete method validation of anagrelide in human plasma was carried out as per US FDA and EMEA guidelines.[14,15] The system suitability test was performed by six repeated injections of aqueous mixture of the analyte and the IS. The carryover experiment was performed to verify any carryover of the analyte and the IS, which may reflect in the subsequent runs. The design of the carryover test comprised of the following sequence of injections, *i.e.* blank plasma sample → six samples of LLOQ → blank plasma sample → ULOQ sample → blank plasma samples, to check for any interference due to carryover. The selectivity of the method was assessed in six different sources of plasma, of which, four were normal K2 EDTA plasma and one each of lipemic and haemolyzed plasma. Sensitivity of the method was assessed by analyzing six sets of spiked plasma samples at the lowest level of the calibration curve concentrations (LLOQ). The matrix effect, expressed as the IS normalized matrix factor (MF), was assessed by comparing the mean area response of post-extraction spiked samples with the mean area of aqueous samples (neat samples) prepared in mobile phase solutions at LQC and HQC levels. The overall precision of the matrix factor was expressed as coefficient of variation (CV).

$$\text{Matrix} = \frac{\text{Peak response area ratio in presence of matrix ions}}{\text{Mean peak response area ratio in absence of matrix ions}}$$

The matrix effect was also evaluated with six different lots of K2 EDTA plasma. Three replicate samples each of LQC and HQC were prepared from different lots of plasma (36 QC samples in total).

The linearity of the method was determined by analysis of five standard calibration curves (CC) containing ten non-zero concentrations. In addition, each curve contains one blank plasma sample and one blank plasma sample with internal standard (zero standard). Each CC was analyzed individually by least squares weighted ($1/x^2$) linear regression. The intra-day accuracy and precision determined using six replicates of LLOQ QC, LQC, MQC1, MQC2 and HQC samples were analyzed along with a calibration curve in a single day. The inter-day accuracy and precision were assessed by analyzing five batches of samples on three consecutive days. The precision (% CV) 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 for LLOQ QC where it must be within ±20%.

Recovery for the analyte and the IS was calculated by comparing the mean detector response of six sets of pre-extraction spiked samples (spiked before extraction) to that of six sets of neat samples (aqueous) at each concentration level. Recovery of anagrelide was determined at a concentration of 0.15 (LQC), 5.08 (MQC2) and 9.07 (HQC) ng mL^{-1}, whereas recovery of the IS was determined at a concentration of 1500 ng mL^{-1}.

Stock solution stability of the analyte and the IS was tested at room temperature for 15 h and at 2–8 °C in a refrigerator for 25 days. The stock solution stability was evaluated by comparing the area response stability samples with the response of the sample prepared from fresh stock solution. The solutions were considered stable if the deviation was within ±10% from the nominal value. Bench-top stability at room temperature (13 h), processed samples stability (autosampler stability for 52 h, wet extract stability for 47 h and reinjection stability for 30 h), and freeze–thaw stability (4 cycles) were evaluated at LQC and HQC levels using six replicates at each level. Similarly, the long term stability of spiked plasma samples stored at −20 ± 5 °C for 26 days and −70 ± 10 °C for 58 days was also studied at both the QC levels. The 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). Drug stability in whole blood at room temperature and at 2–8 °C in a refrigerator for 3 h was also determined by spiking blood samples with the analyte at both the QC levels.

The method ruggedness was verified by analyzing one precision and accuracy batch on a different column of the same make (different batch no.) using a different set of reagents processed by a different analyst. Dilution reliability was performed to extend the upper concentration limit with acceptable precision and accuracy. Six replicates each at a concentration of about 1.66 times of the uppermost calibration standard were diluted to half and quarter with screened human blank plasma. The diluted samples were processed and analyzed with un-diluted calibration curve samples.

Pharmacokinetic study protocol and incurred samples' reanalysis

The proposed method was applied to determine the anagrelide plasma concentration for a pharmacokinetic study conducted in 6 healthy Indian subjects. Healthy South Indian male subjects with the age group of 20–40 years, the body-mass index (BMI) between≥18.5 kg m^{-2} and ≤24.9 kg m^{-2}, and the body weight not less than 50 kg were selected for the study. All the volunteers provided the written informed consent and were fasted for 12 h before the drug formulation administration. The subjects were orally administered with a single dose of anagrelide hydrochloride (0.5 mg capsule) with 200 mL of water. Blood samples were drawn at pre-dose and 0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75, 2, 2.5, 3, 4, 5, 6, 8, 10, 12 and 16 h and collected in K_2 EDTA vacutainer (5 mL) collection tubes (BD, Franklin, NJ, USA). The tubes were centrifuged at 3200 rpm for 10 min and plasma was collected. The collected plasma samples were stored at −70 ± 10 °C until use. Plasma samples were spiked with the IS and processed as per the extraction procedure described earlier. The main pharmacokinetic parameters of anagrelide were calculated by a non-compartmental model using WinNonlin Version 5.2. An ISR was also performed by selecting 12 subject samples (2 samples from each subject) near C_{max} and the elimination phase in the pharmacokinetic profile of the drug. The ISR values were compared with the initial values. The percent change in the value should not be more than ±20%.[16,17]

Results and discussion

Mass spectrometry

The present method was developed using ESI as the ionization source in the positive ionization mode. Initially, the analyte and the IS were tuned in positive and negative ionization modes using tuning solution (50 ng mL^{-1}), but the response obtained in positive mode was much higher than in negative mode. The protonated form of the analyte and the IS, $[M + H]^+$ ion, was the precursor ion in the Q_1 spectrum and was used as the precursor ion to obtain Q_3 product ion spectra. The most sensitive mass transition was observed from *m*/*z* 256.0 to 199.1 for anagrelide and from *m*/*z* 267.4 to 226.2 for the IS. The most intense and consistent product ion in Q3 MS spectra of the analyte and the IS was obtained by optimizing the collision energy and collision cell exit potential. The source parameters like nebulizer gas (GS1), auxiliary gas (GS2), collision gas, temperature and ion spray voltage were optimized to obtain adequate and reproducible response for the analyte. The dwell time for each transition was set at 200 ms. The product ion mass spectra of anagrelide are presented in Fig 2. As previous publications have discussed the details of fragmentation patterns of nevirapine,[18] we are not presenting the data pertaining to this. The LC-MRM technique was chosen for the assay development due to its inherent selectivity and sensitivity.[19]

Chromatography

Various mobile phase compositions of acetonitrile/methanol with acidic modifiers like formic acid, acetic acid, ammonium acetate and ammonium formate were tested in different volume ratios. The response obtained with methanol and 5 mM ammonium acetate as the mobile phase was satisfactory, but not reproducible. In addition, a choice of chromatographic columns like C_8 and C_{18} of different makes (Zorbax SB C_{18}, 50 × 4.6, 5 μm; Zorbax XDB-phenyl 75 × 4.6, 3.5 μm; Kromasil 100-$5C_{18}$, 100 × 4.6, 5 μm; Ace $3C_{18}$ 150 × 4.6, 3 μm; Alltima HP C_{18} 50 × 4.6, 3 μm; Hypurity advance 75 × 4.6, 5 μm; Discovery HS C_{18} 50 mm × 4.6 mm, 5 μm) were tested to achieve adequate retention time with a short run time, better separation from endogenous components, symmetric peak shape and satisfactory response for the analyte. The best chromatographic conditions were achieved with methanol and 0.1% formic acid in 5 mM ammonium acetate (80 : 20, v/v) as the mobile phase under isocratic conditions. The Discovery® HS C_{18} (50 × 4.6, 5 μm) column gave a good peak shape and adequate response even at the lowest concentration (0.05 ng mL^{-1}) level for the analyte. In addition, the effect of flow rate was also studied from 0.3 to 1.0 mL min^{-1}, which was also responsible for the acceptable chromatographic peak shape and short run time and finally the flow rate was set at 1.0 mL min^{-1}. The retention times of the analyte and the IS obtained with the above optimized chromatographic conditions were low enough (0.80 and 0.65 min) allowing a short run time of 2.0 min.

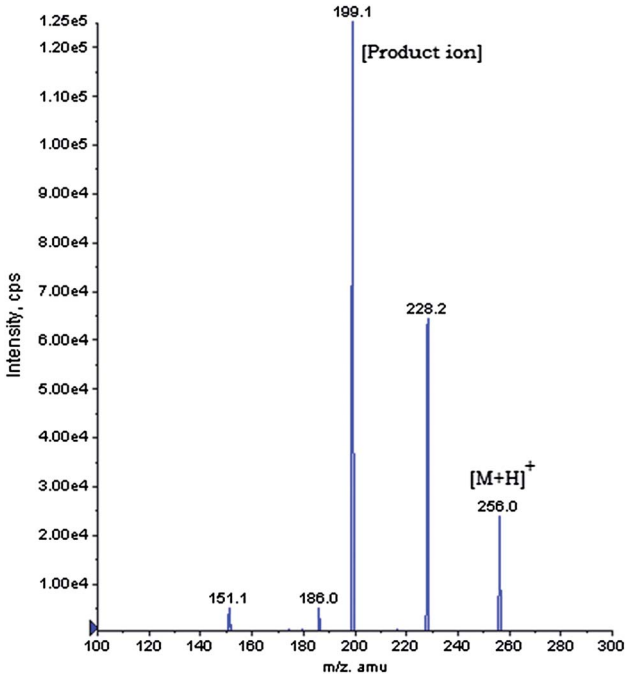


Fig. 2 Product ion mass spectra of $[M + H]^+$ of anagrelide.

Extraction procedure optimization

Earlier, authors[3,9,10] have employed liquid–liquid extraction to extract anagrelide from human plasma. Thus, protein precipitation (PP) was attempted with methanol/acetonitrile, but the recovery was inconsistent for the analyte at LQC concentration. Clean samples are essential for minimizing ion suppression

and the matrix effect on LC-MS/MS analysis. SPE is helpful in producing a clean sample with no or a minimal matrix effect. Also, there are no LC-MS/MS reports based on SPE for determination of anagrelide in the literature so far. Therefore, SPE was tried with Oasis HLB, Strata and Orpheus C_{18} extraction cartridges with/without acidic buffer addition to obtain the clean sample and to remove the interference from endogenous components. But although Oasis HLB cartridges gave promising results, the recovery was not consistent at the lowest level. Finally, promising results were obtained with Strata-X 33 μm polymeric sorbent (30 mg/1 mL) extraction cartridges, which can produce a clean chromatogram for a blank sample and yields the maximum recovery for the analyte from plasma. Use of the mobile phase as an eluting solvent helped in achieving reproducible and quantitative recovery of the analyte and avoided drying and reconstitution steps.

Selection of internal standard

A perfect internal standard should impersonate the analyte during ionization, chromatography and extraction. Ideally, stable labeled isotope drugs are preferably used in LC-MS/MS analysis. But these compounds are expensive and/or not available to serve as an internal standard. So, at the initial stages of this work, many compounds were investigated in order to find a suitable IS, and finally nevirapine was selected, based on chromatographic elution, ionization and extraction efficiency. Moreover, the current validation results encouraged its selection as an internal standard.

System suitability and carryover test

The precision (%CV) for the system suitability test was in the range of 0.05–0.51% for the retention time and 0.2–1.10% for the area response of the analyte and IS. Carryover evaluation was performed to ensure that it does not affect the accuracy and precision of the proposed method. No significant carryover was observed in the blank sample after injecting the highest concentration of the analyte (ULOQ, upper limit of quantitation), which indicates no carry-over of the analyte in subsequent samples (data not shown).

Selectivity

The selectivity of the method was examined by analyzing a blank human plasma extract (Fig. 3A) and an extract spiked only with the IS (Fig. 3B). As shown in Fig. 3A, no significant direct interference in the blank plasma traces was observed from endogenous substances in drug-free human plasma at the retention time of the analyte and the IS. Similarly, Fig. 3B shows the absence of direct interference from the IS to the MRM channel of the analyte. Fig. 3C depicts a representative ion-chromatogram for the LLOQ QC sample (0.05 ng mL^{-1}). A representative chromatogram

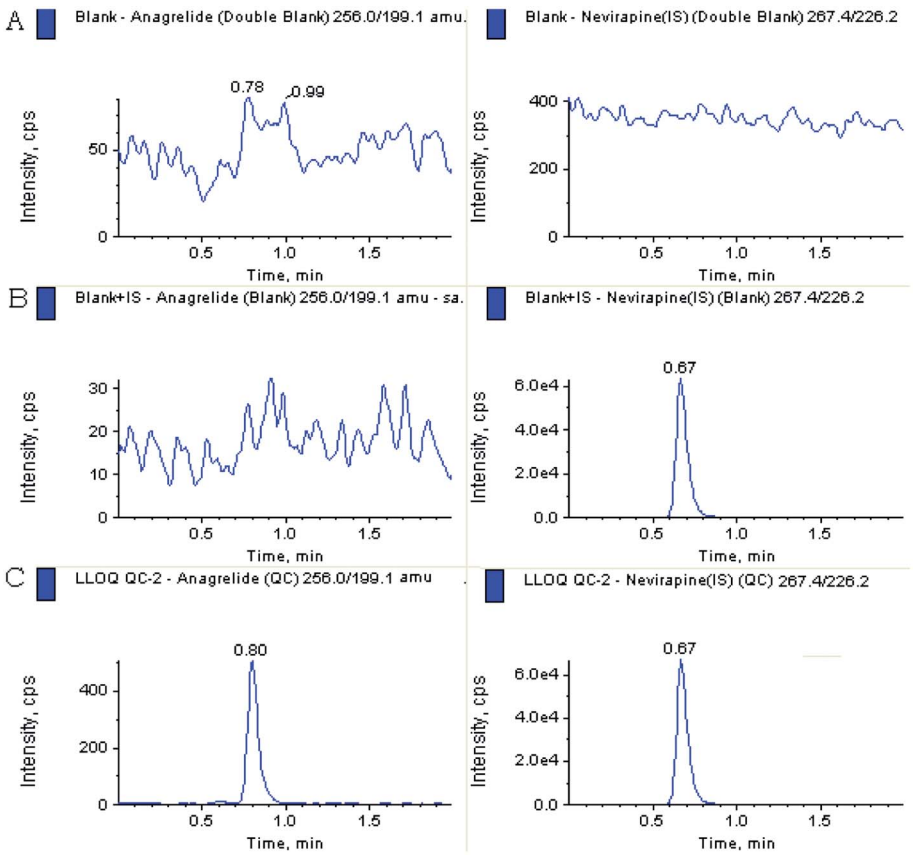


Fig. 3 Typical MRM chromatograms of anagrelide (left panel) and IS (right panel) in human blank plasma (A), and human plasma spiked with IS (B), and a LLOQ QC sample along with IS (C).

resulting from the analysis of a subject blank plasma sample and a 0.5 h subject plasma sample after administration of a 0.5 mg oral single dose of anagrelide is shown in Fig. 4.

Matrix effect

Matrix effect assessment was done with the aim of checking the effect of different lots of plasma on the back calculated value of QC's nominal concentration. The results obtained were well within the acceptable limits as shown in Table 1. No significant matrix effect was observed in all the six batches of human plasma for the analyte at low and high quality control concentrations. Also, the extraction method was rugged enough and gave accurate and consistent results when applied to real subject samples.

Sensitivity

The lowest limit of reliable quantification for the analyte was set at the concentration of LLOQ (0.05 ng mL^{-1}). The signal-to-noise ratio (S/N) was measured at this concentration and found to be $\geq$10. The precision and accuracy at LLOQ concentration were found to be 2.96% and 93.0%, respectively.

Linearity, precision and accuracy

The analyte showed good linearity in the concentration range of 0.05–10.0 ng mL^{-1}. Both the regression models ($1/x$ and $1/x^2$) were compared and the best fit for the concentration-detector response relationship was obtained with a weighting factor of $1/x^2$. The mean correlation coefficient values were in the range of 0.9990–0.9998 for all the analytical runs generated during the entire course of validation.

The intra-day and inter-day precision and accuracy results in plasma QC samples are summarized in Table 2. The precision (% CV) and accuracy values of anagrelide for intra- and inter-day ranged from 0.83–4.31% and 92.0–102%, and 1.10–6.25% and 94.6–103%, respectively. The results revealed good precision and accuracy.

Extraction efficiency

The mean overall recovery of anagrelide was 92.6 $\pm$ 2.32% with the precision range of 4.08–7.52% and the recovery of IS was 68.9% with the precision range of 1.07–6.61%. Good and reproducible recoveries were obtained for the analyte and the IS. Thus, the assay has been proved to be robust in high throughput bioanalysis.

Stability studies and dilution integrity

Analyte stability under various conditions was evaluated. In the different stability experiments carried out *viz*. bench top stability (13 h), autosampler stability (52 h), wet extract stability

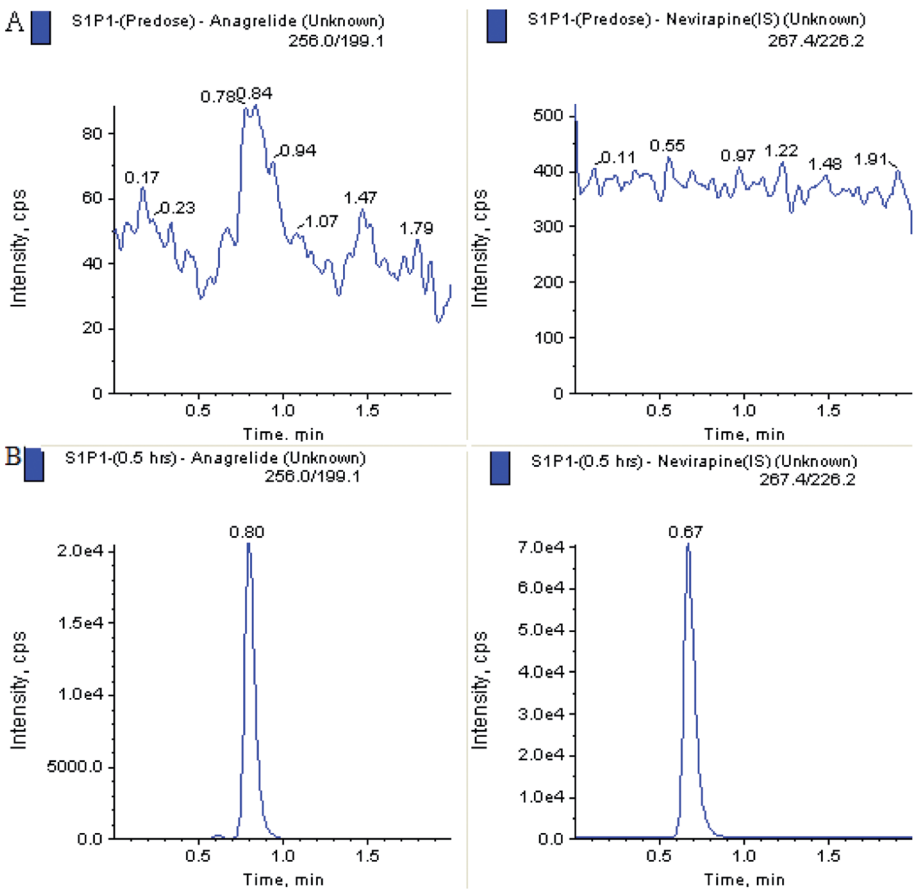


Fig. 4 MRM chromatograms resulting from the analysis of subject blank plasma sample (A) and 0.5 h subject plasma sample (B), after the administration of a 0.5 mg oral single dose of anagrelide capsule. The sample concentration was determined to be 1.75 ng mL^{-1}.

Table 1 Matrix effect of anagrelide in human plasma ($n = 3$)

	LQC (0.15 ng mL^{-1})			HQC (9.07 ng mL^{-1})		
Plasma lot	Concentration found (mean ± SD; ng mL^{-1})	% Accuracy	IS-normalized MF	Concentration found (mean ± SD; ng mL^{-1})	% Accuracy	IS-normalized MF
Lot 1	0.16 ± 0.003	102	1.03	9.28 ± 0.05	102	1.00
Lot 2	0.16 ± 0.002	103	1.04	9.17 ± 0.03	101	1.01
Lot 3	0.16 ± 0.004	107	1.00	9.18 ± 0.13	101	1.01
Lot 4	0.16 ± 0.000	103	1.01	9.37 ± 0.15	103	1.00
Lot 5	0.16 ± 0.006	107	0.99	9.12 ± 0.09	101	1.01
Lot 6	0.16 ± 0.005	102	0.99	9.27 ± 0.07	102	1.02

Table 2 Precision and accuracy data for anagrelide[a]

Quality control	Run	Concentration found (mean ± SD; ng mL^{-1})	Precision (%)	Accuracy (%)
Intra-day variations (six replicates at each concentration)				
LLOQ	1	0.05 ± 0.002	4.53	93.5
	2	0.05 ± 0.002	3.73	90.5
	3	0.05 ± 0.002	4.21	102
	4	0.05 ± 0.002	4.04	92.2
	5	0.05 ± 0.004	7.25	94.8
LQC	1	0.16 ± 0.004	2.48	103
	2	0.15 ± 0.003	1.64	100
	3	0.16 ± 0.005	2.86	106
	4	0.15 ± 0.004	2.42	99.2
	5	0.16 ± 0.005	3.28	104
MQC1	1	1.25 ± 0.01	0.79	102
	2	1.24 ± 0.02	1.86	102
	3	1.24 ± 0.02	1.82	102
	4	1.16 ± 0.03	2.44	95.5
	5	1.27 ± 0.06	4.93	104
MQC2	1	5.02 ± 0.04	0.76	98.8
	2	5.01 ± 0.05	0.95	98.6
	3	4.99 ± 0.08	1.67	98.2
	4	4.78 ± 0.05	1.03	94.2
	5	4.96 ± 0.04	0.76	97.6
HQC	1	8.89 ± 0.05	0.61	98.0
	2	8.94 ± 0.10	1.17	98.6
	3	8.95 ± 0.09	1.05	98.7
	4	8.94 ± 0.12	1.40	98.6
	5	8.84 ± 0.08	0.87	97.4
Inter-day variations (30 replicates at each concentration)				
LLOQ		0.05 ± 0.003	6.25	94.6
LQC		0.16 ± 0.005	3.41	103
MQC1		1.23 ± 0.05	3.96	101
MQC2		4.95 ± 0.10	2.05	97.5
HQC		8.91 ± 0.10	1.10	98.3

[a] Spiked concentrations of LLOQ, LQC, MQC1, MQC2 and HQC are 0.05, 0.15, 1.22, 5.08 and 9.07 ng mL^{-1}, respectively.

(47 h), repeated freeze-thaw cycles (4 cycles), reinjection stability (30 h) and long term stability at −70 °C for 58 days, the mean % nominal values of the analyte were found to be within ±15% of the predicted concentrations for the analyte at its LQC and HQC levels (Table 3). Therefore, the results were found to be within the acceptable limits during the entire validation.

Stock solutions of anagrelide and IS were found to be stable for 25 days at 2–8 °C. The percentage stability (with the precision range) of anagrelide and IS was 103% (0.78–2.95%) and 102% (0.97–2.99%), respectively. For whole blood sample stability at room temperature and at 2–8 °C in a refrigerator for 3 h, the percentage change from comparison samples was less than 1.09% at two QC levels.

The upper concentration limit of anagrelide can be extended to 16.6 ng mL^{-1} by using half (1 : 2) or quarter (1 : 4) dilution with screened human blank plasma. The precision (%CV) for dilution integrity of 1/2 and 1/4 dilution was found to be 2.20% and 1.32%, while the accuracy results were found to be 94.3% and 96.4%, respectively.

Method ruggedness

For method ruggedness of anagrelide, the precision (%CV) and accuracy values with different analysts, with different columns (different batch no.) and with different sets of reagents ranged from 1.05–4.21% and 98.2–106%, respectively.

Pharmacokinetic study and incurred samples reanalysis

The proposed method was successfully used to quantify anagrelide plasma concentration for a pharmacokinetic study in healthy South Indian adult male subjects ($n = 6$). Fig. 5 depicts the mean plasma concentration *vs.* time profile of anagrelide after administration of a single 0.5 mg oral dose of anagrelide under fasting conditions. Table 4 summarizes the mean pharmacokinetic parameters of anagrelide.

The reproducibility of the present method was established by reanalysis of incurred samples (ISR). For the incurred samples' analysis two plasma samples from each subject were selected and re-assayed in a single bioanalytical run. The differences in concentrations between the ISR and the initial values for all the tested samples were less than 20% (Table 5), indicating good reproducibility of the present method.

Comparison with earlier reports

Until now, only three analytical methods have been reported for the determination of anagrelide in biological samples.[3,9,10]

The proposed LC-MS/MS method has the highest sensitivity compared to earlier reports (Kerns *et al.*, 1987)[9] and equally sensitive with those of Zhu *et al.*, (2005)[10] and Martínez-Sellés

Table 3 Stability data for anagrelide in plasma ($n = 6$)

Stability test	QC (spiked concentration, ng mL^{-1})	Mean ± SD (ng mL^{-1})	Accuracy/stability (%)	Precision (%)
Autosampler stability (at 15 °C for 52 h)	0.15	0.16 ± 0.005	104	3.15
	9.07	8.93 ± 0.09	98.4	0.99
Wet extract stability (at room temperature for 47 h)	0.15	0.16 ± 0.004	102	2.29
	9.07	8.98 ± 0.07	99.0	0.74
Bench top stability (13 h at room temperature)	0.15	0.16 ± 0.004	103	2.61
	9.07	8.97 ± 0.07	99.0	0.76
Freeze–thaw stability (four cycles)	0.15	0.16 ± 0.003	103	1.93
	9.07	8.99 ± 0.10	99.1	1.09
Reinjection stability (30 h)	0.15	0.15 ± 0.003	101	2.05
	9.07	8.90 ± 0.05	98.2	0.60
Long-term stability (at −70 °C for 58 days)	0.15	0.15 ± 0.004	102	2.77
	9.07	8.78 ± 0.14	96.8	1.58
Long-term stability (at −20 °C for 26 days)	0.15	0.16 ± 0.004	103	2.51
	9.07	8.90 ± 0.06	98.2	0.64

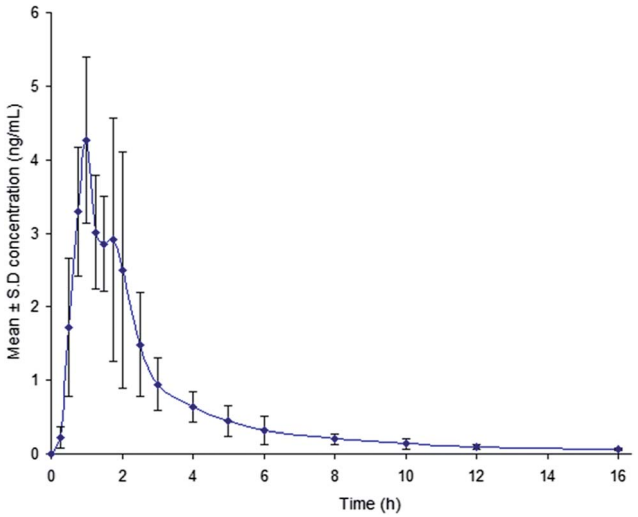


Fig. 5 Mean plasma concentration–time profile of anagrelide in human plasma following oral dosing of anagrelide (0.5 mg capsule) to healthy volunteers ($n = 6$).

Table 4 Pharmacokinetic parameters of anagrelide after single oral administration of a 0.5 mg anagrelide capsule to healthy South Indian male subjects ($n = 6$, mean ± SD)

Parameter	Mean ± SD
C_{max} (ng mL^{-1})	4.95 ± 0.50
t_{max} (h)	1.29 ± 0.46
AUC_{0-t} (ng h mL^{-1})	9.59 ± 2.45
AUC_{0-inf} (ng h mL^{-1})	9.95 ± 2.49
$t_{1/2}$ (h)	4.41 ± 1.53
Kel (h^{-1})	0.17 ± 0.05

et al., (2013)[3] with the use of smaller plasma volume (100 μL). All the reported methods[3,9,10] have employed liquid–liquid extraction, drying, evaporation and reconstitution for sample preparation. But, the present method utilizes a simple SPE technique

Table 5 Incurred samples re-analysis data of anagrelide

Sample	Initial conc. (ng mL^{-1})	Re-assay conc. (ng mL^{-1})	Difference[a] (%)
1	3.47	3.93	−12.3
2	0.17	0.17	−4.15
3	3.67	3.65	0.55
4	0.19	0.18	6.59
5	3.47	3.35	3.49
6	0.22	0.20	9.71
7	4.62	4.80	−3.70
8	0.25	0.25	−2.81
9	3.81	3.38	12.0
10	0.16	0.15	5.06
11	3.25	3.09	5.20
12	0.21	0.24	−11.7

[a] Expressed as [(initial conc. − re-assay conc.)/average] × 100%.

with direct elution (avoids drying, evaporation and reconstitution steps) for sample preparation, thereby significantly reducing the sample processing time. The ISR results obtained demonstrated that the present method is highly reproducible and suitable for pharmacokinetic/bioequivalence studies in humans.

Conclusions

The LC-MS/MS assay reported here is simple, rapid, specific and sensitive for quantification of anagrelide in human plasma and is fully validated according to commonly acceptable FDA guidelines. The simple solid-phase extraction method gave consistent and reproducible recoveries for the analyte from human plasma. This is the first LC-MS/MS report for the determination of anagrelide based on the SPE technique for sample preparation. This method provided good linearity. The stability of the analyte in plasma and in aqueous samples under different conditions has been extensively evaluated. A sample turnover rate of less than 2.0 min makes it an attractive

procedure in high-throughput bioanalysis of anagrelide. This method was found to be reliable and reproducible to support pharmacokinetic studies in humans. From the results of all the validation parameters, we can conclude that the developed method can be useful for bioavailability and bioequivalence (BA/BE) studies and routine therapeutic drug monitoring with the desired precision and accuracy.

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