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High-performance liquid chromatography–mass spectrometry method for determination of anagrelide in human plasma

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Abstract

This paper describes a simple, fast and sensitive liquid chromatography–mass spectrometry method for quantification of an anti-thrombocythemic agent, anagrelide in human plasma. The samples were subjected to a liquid–liquid extraction after addition of a buffer and an internal standard. Chromatography was performed on an Inertsil ODS2 column and the extract was injected onto a HPLC system coupled with mass spectrometric detection. Linear responses for standards were observed from 50 to 7500 pg/ml. The accuracy of intra-assay and inter-assay were in the ranges 4.3–4.4% and 4.8–5.6%, respectively. The method is simple and reproducible with a run time of less than 2 min.

Keywords: HPLC; Anagrelide; Human

1. Introduction

Essential thrombocythemia is a chronic myeloproliferative disorder characterized by marked megakaryocytic hyperplasia with sustained thrombocytosis. Anagrelide hydrochloride was the first drug approved by the FDA in 1997 for the treatment of this disorder and was designated an orphan drug. It was specifically indicated for the treatment of patients with essential thrombocythemia to reduce the elevated platelet count and the risk of thrombosis and to ameliorate associated symptoms [1]. It was believed that the response to anagrelide was associated with decreased megakaryocyte frequency. The reduction in cell number induced by anagrelide therapy was associated with a concomitant decrease in megakaryocyte size and ploidy [2]. Historically, radioactive phosphorus, alkylators (busulfan, pipobroman), hydroxyurea and interferonalfa have been the mainstay of therapy; however, these agents may cause serious adverse effects [3]. To determine practice patterns in the management of polycythemia vera, the US American Society of Hematology conducted a survey. Results indicated that hydroxyurea (62.8%) and anagrelide (35.4%) were the primary agents used to treat thrombocytosis [4]. Now, anagrelide has been established as an alternative first-line therapy to reduce the platelet count, when indicated, in clonal thrombocytosis [5,6]. Phase III clinical trials (PT1 and ANAHYDRET) are underway to directly compare the efficacy and safety of anagrelide and hydroxyurea [7]. With increased interest in this product, it is necessary to have a simple analysis procedure to meet the needs of different studies.

Our literature search uncovered only one paper that was published in 1987, which described the determination of anagrelide in plasma [8]. The method was by capillary gas chromatography–mass spectrometry. The sample preparation involved a liquid–liquid extraction followed by a derivatization, which is a complicated and labor-intensive procedure. The linear range of the method was 0.5–100 ng/ml. Due to the complexity of the GC method and the challenge of attaining the required sensitivity for accurate assessment of the pharmacokinetic parameters of anagrelide, LC/MS/MS technology becomes a simpler and better solution for the determination of anagrelide. The aim of the present work is to have a simple, fast, sensitive and reproducible method

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for determination of anagrelide in human plasma using high-performance liquid chromatography–mass spectrometry method with electro-spray ionization in positive ionization mode.

2. Experimental

2.1. Materials and reagents

The anagrelide hydrochloride monohydrate and clonazepam were obtained from Pharmascience (Montreal, Canada). The methanol, acetonitrile, formic acid, cyclohexane and borate pH 10 buffer solutions were purchased from Fisher Scientific (Nepean, Canada). The HPLC-grade water was obtained from a laboratory Nano-Pure water purification system.

Drug-free human plasma was used for the preparation of the calibration standards. The quality control samples were purchased from Biological Specialties (Colmar, PA, USA). All plasma samples were stored at −20 °C prior to usage.

2.2. Stock solutions and standards

The anagrelide and internal standard stock solutions were prepared in methanol at a concentration of 100 μg/ml and they were stored at −20 °C. The structures of anagrelide and clonazepam (internal standard) are presented in Figs. 1 and 2. A seven-point calibration standard curve of anagrelide ranging from 50 to 7500 pg/ml was prepared by spiking the drug-free human plasma containing EDTA with an appropriate amount of anagrelide. The quality control samples at three concentration levels (150, 3000 and 5000 pg/ml) were prepared in a similar manner. Drug-free human plasma was tested before spiking to ensure that no endogenous interference was found at the anagrelide and internal standard retention times.

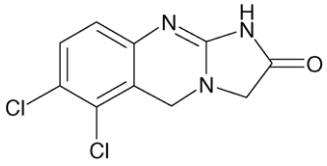

Fig. 1. Chemical structure of anagrelide.

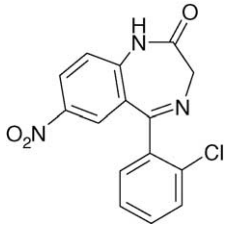

Fig. 2. Chemical structure of clonazepam.

Table 1
Summary of anagrelide calibration standards

Concentration added (pg/ml)	Concentration found (pg/ml)	R.E.[a] (%)	CV (%)	n
50	50.40	0.8	2.2	5
100	98.18	−1.8	4.5	5
500	503.66	0.7	4.1	5
2000	1988.56	−0.6	2.5	5
4000	3993.02	−0.2	1.7	5
6000	6039.40	0.7	1.4	5
7500	7524.60	0.3	3.1	5

Correlation coefficient = 0.99860 ± 0.0013 (n = 5).
[a] R.E. = relative error.

2.3. Extraction procedure for plasma samples

A 0.5-ml aliquot of human plasma was placed into a screw cap glass tube, followed by 0.1 ml of internal standard (20 ng/ml of clonazepam in 1:1 methanol/H_2O solution) and by 0.5 ml of pH 10 buffer solution. The mixture was vortexed and 7 ml of extraction solvent, cyclohexane, was then added. The mixture was shaken and centrifuged at 3000 rpm for 10 min. The organic layer was evaporated to dryness under a nitrogen evaporator at 40 °C, reconstituted in 0.15 ml of mobile phase and 10 μL was injected into the LC/MS/MS for analysis.

2.4. Chromatography and quantification

Chromatographic separation was carried out on an Agilent 1100 HPLC with an Inertsil ODS2 column (50 mm × 2.1 mm, 5 μm) that was purchased from Chromatography Sciences (St.-Laurent, Québec, Canada). A mobile phase consisting of acetonitrile, methanol and 1.0% formic acid (5:3:2, v/v/v) was used with a flow-rate of 0.2 ml/min. The total run time for each sample analysis was 2.0 min.

A mass spectra was obtained by using a TSQ Quantum mass spectrometer (Thermo Finnigan, USA) equipped with an ion spray source. The data acquisition was ascertained by Xcalibur software. Positive mode was used for the analysis. The mass ion-pair measured was 256.1 → 199.1 for anagrelide and 316.0 → 270.1 for the internal standard. The mass spectrometer conditions were set as follows: the spray voltage

Table 2
Assay variability of anagrelide in human plasma

Concentration added (pg/ml)	Concentration found (pg/ml)	R.E.[a] (%)	CV (%)	n
Inter-day				
150	158.41	5.6	6.4	18
3000	3145.67	4.9	2.1	18
5000	5241.24	4.8	2.9	18
Intra-day				
50	52.25	4.5	11.1	6
150	156.65	4.4	7.5	6
3000	3129.62	4.3	1.7	6
5000	5214.77	4.3	1.3	6

[a] R.E. = relative error.

at 4500 V, the sheath gas at 50 psi and the capillary temperature at 375 °C. The quantification of anagrelide in human plasma was based on the peak area ratios of anagrelide versus the internal standard.

2.5. Validation

The method has been validated for accuracy, precision, selectivity, linearity, recovery and stability. The accuracy was determined by replicating the analysis of samples containing known amounts of analyte. The intra-assay precision and accuracy were determined with six replicates of the lower limit of quantification (LLOQ) and quality control samples that were extracted from the same batch. The inter-assay precision and accuracy were determined by analyzing the quality control samples that were tested on five different occasions. The inter- and intra-assay precision and accuracy evaluations were based on back-calculated concentrations. The acceptance criteria was ±15% for all assessments.

The selectivity was determined by analyzing blank plasma samples from different donors to test for interference with the anagrelide and internal standard retention times.

The recovery of anagrelide and the internal standard was determined by comparing the peak area response of extracted analytes with the peak area response of solutions that were prepared at the same concentration (150, 3000 and 5000 pg/ml) in the mobile phase.

The extracted plasma sample stability, bench top stability, in-process stability, freeze–thaw stability and frozen plasma sample stability were evaluated. All stability evaluations were based on back-calculated concentrations. The stability evaluations involved an analysis of the low, mid and high quality control samples with the freshly prepared standard curve in comparison with the stability samples. In addition, the method was validated for the plasma matrix effect by comparing the responses of the anagrelide and internal standard in plasma from 6 different donors.

3. Results

3.1. Limit of quantification, linearity and precision

The lower limit of quantification (LLOQ) for anagrelide in human plasma is 50 pg/ml and the typical signal/noise ratio at

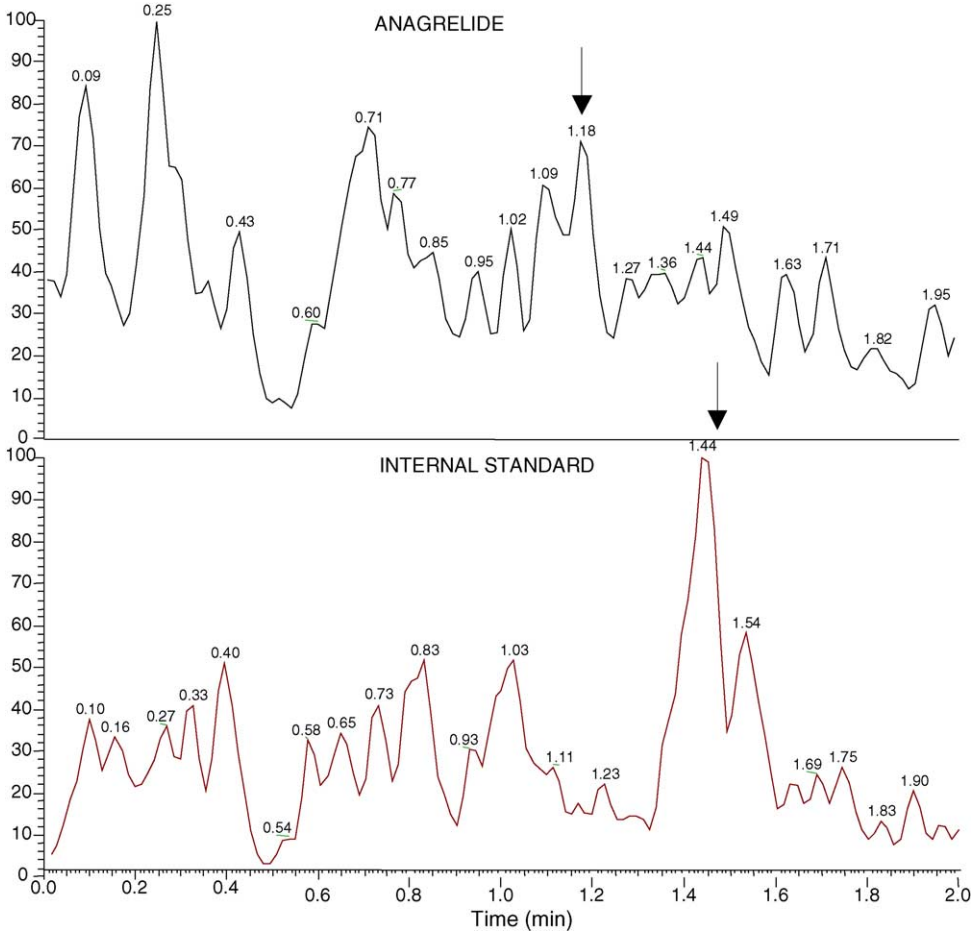

Fig. 3. Representative chromatograms of extracted blank plasma samples.

this concentration was at least 10. The calibration curves are linear in the concentration range 50–7500 pg/ml with an average regression of 0.99860 ± 0.0013 ($n = 5$). The results for the anagrelide calibration samples are presented in Table 1. A weighted least square regression analysis using the method of least squares was performed ($y = mx + b$).

Inter-assay precision was determined by analyzing five calibration curves with quality control samples on different days. The intra-assay precision was determined by analyzing six replicates of quality control samples extracted from the same batch. The results of inter- and intra-day precisions for anagrelide in human plasma are tabulated in Table 2. For quality control samples at a concentration of 150, 3000 and 5000 pg/ml, intra- and inter-assay precisions were between 1.3 and 7.5%.

3.2. Selectivity

A representative chromatogram of extracted blank plasma is presented in Fig. 3. Representative chromatograms of extracted plasma samples containing 50 pg/ml (low standard) and 7500 pg/ml (high standard) of anagrelide are presented in Figs. 4 and 5. Six different sources of drug-free human plasma samples were screened and no significant interference was observed at the anagrelide and internal standard retention times.

3.3. Recovery and stability

The overall extraction yield of anagrelide was 64.2% and the extraction yield of the internal standard was 87.5%. The evaluation of anagrelide recovery was obtained from the average of six replicates of low, mid and high quality control samples. The CV% for the anagrelide recovery was 6.4, 2.2, 1.1 and 2.9 for the low, mid, high QC sample and internal standard respectively.

Three replicates of low, mid and high quality control samples were used for the stability tests. Both bench top stability and in-process stability tests showed that anagrelide in human plasma is stable for at least 2 h at room temperature.

Extracted anagrelide and internal standard are stable in the auto-sampler at $4 \pm 2\,^{\circ}C$ for 68 h. This processed sample stability was evaluated by comparing the extracted plasma samples that were injected immediately (time 0) with the samples that were re-injected after sitting in the auto-sampler at $4 \pm 2\,^{\circ}C$ for 68 h.

The freeze–thaw stability indicated that anagrelide is stable for at least three freeze–thaw cycles. Results of frozen

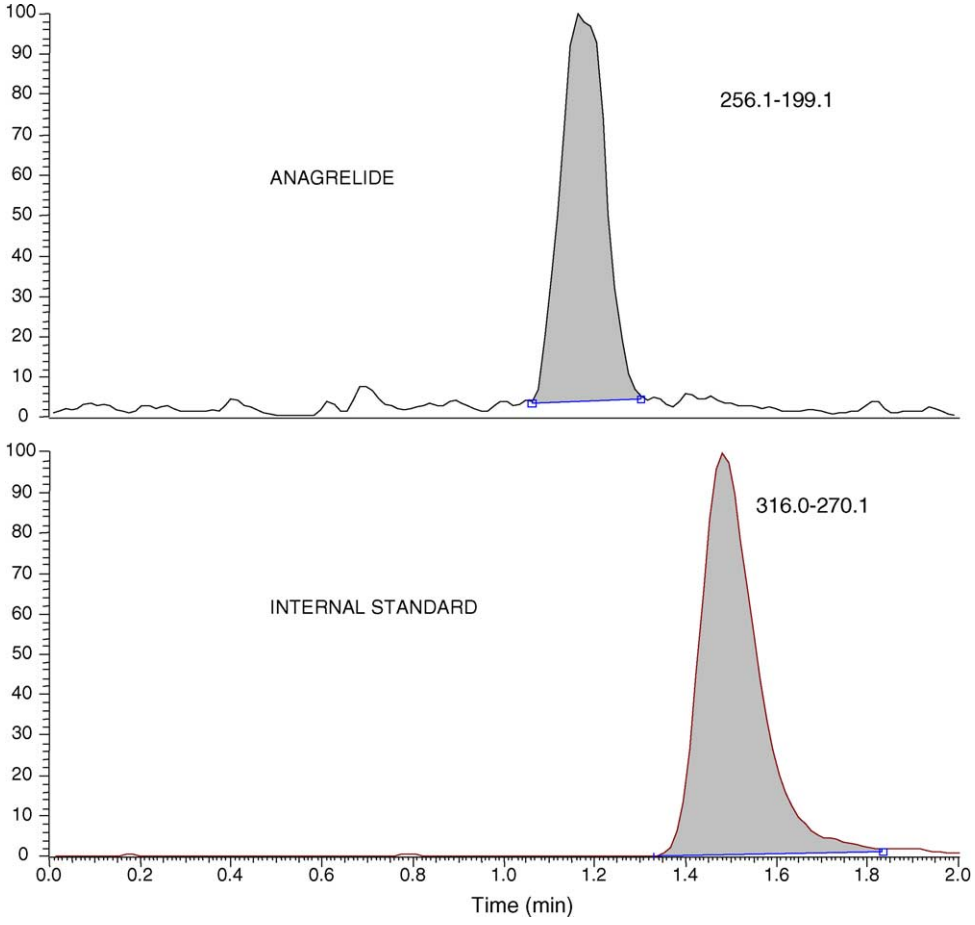

Fig. 4. Representative chromatogram of extracted plasma samples (LLOQ).

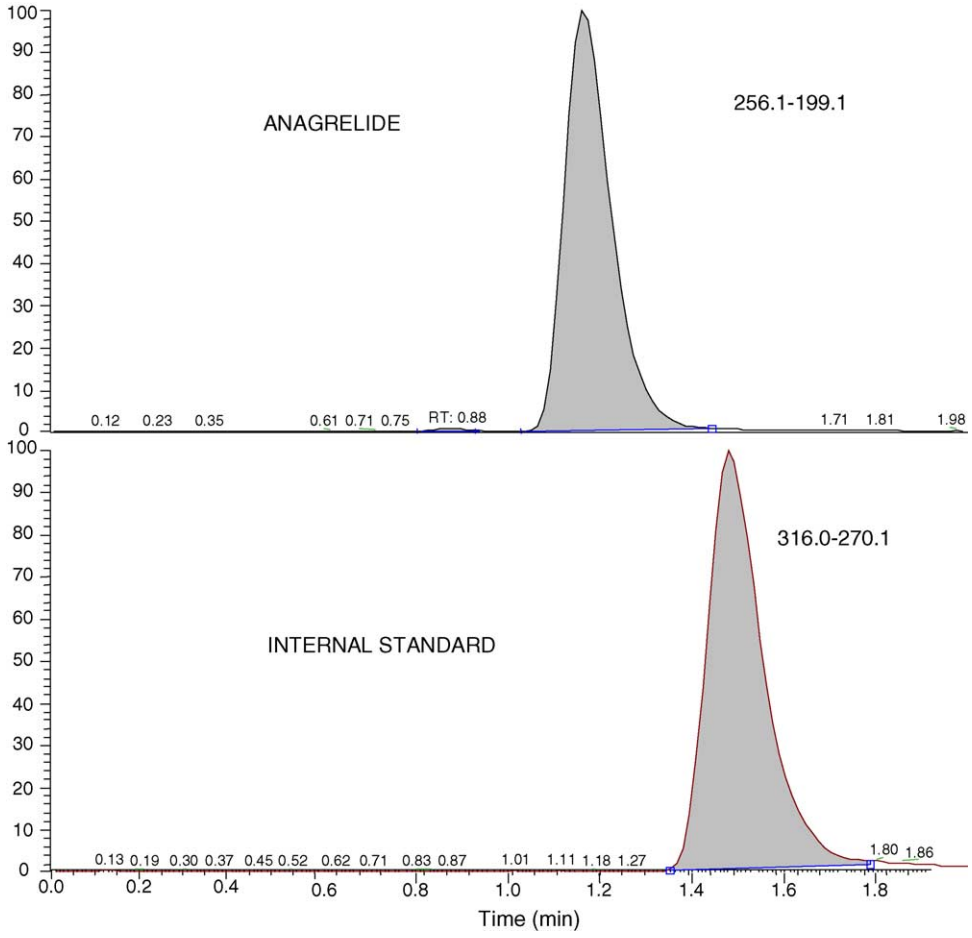

Fig. 5. Representative chromatogram of extracted plasma samples containing 7500 pg/ml anagrelide.

sample stability for anagrelide in human plasma showed that it was stable for at least 56 days at $-20 \pm 5\,^{\circ}C$. The long-term stability evaluation involved a comparative analysis of the low, mid and high quality control stability samples together with freshly spiked standards and quality control samples.

Table 3
Matrix effect of anagrelide in human plasma

Concentration added (pg/ml)	Concentration found (pg/ml)	R.E.[a] (%)	CV (%)	*n*
Matrix #1				
150	168.93	12.6	0.8	3
3000	3331.47	11.0	0.9	3
5000	5463.77	9.3	1.3	3
Matrix #2				
150	157.30	4.9	1.3	3
3000	3238.73	8.0	0.3	3
5000	5289.10	5.8	1.0	3
Matrix #3				
150	166.50	11.0	4.3	3
3000	3273.03	9.1	0.5	3
5000	5539.43	10.8	2.4	3

[a] R.E. = relative error.

A matrix effect study indicated that the response of anagrelide and the internal standard are consistent in plasma from different donors. The precision of the quality control samples is between 0.3 and 4.3%. The accuracy is between 4.9 and 12.6%. The matrix effect verification was evaluated with the preparation of quality control samples (150, 3000 and 5000 pg/ml) in matrix from different donors and the concentrations were back-calculated from the calibra-

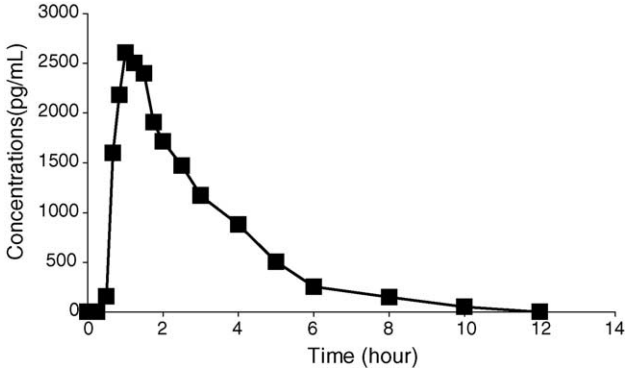

Fig. 6. Representative concentration time profile following an oral dose of anagrelide capsule to human volunteer.

tion curve. The results of the matrix effect are presented in Table 3.

3.4. Application

This method has been applied to a bioavailability study in healthy volunteers. The blood samples were collected from the volunteers over a 12-h period following an oral dose of anagrelide capsules. The plasma samples from the study were stored at −20 °C until analysis. A representative profile of the anagrelide plasma concentration versus sampling time is presented in Fig. 6.

4. Conclusions

This is the first LC/MS/MS method for the determination of anagrelide in human plasma. The method consists of a simple liquid–liquid extraction procedure and isocratic chromatography conditions. High-performance liquid chromatography coupled with positive ion tandem mass spectrometry detection is a method, which allows for a very low limit of detection. This method has excellent sensitivity and reproducibility. It is simple and has a run time of less than 2 min.

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