Asian Journal of Chemistry; Vol. 36, No. 2 (2024), 325-330

ASIAN JOURNAL OF CHEMISTRY

https://doi.org/10.14233/ajchem.2024.30785

Impurity Profiling and a Stability Indicating Advanced Liquid Chromatographic Method Development and Validation for the Estimation of Related Impurities of Anagrelide

PAVANI PEDDI[1,*], P.T.S.R.K. PRASADA RAO[2], CHEBOLU NAGA SESHA SAI PAVAN KUMAR[3], NANNAPANENI USHA RANI[1] and S. LAKSHMI TULASI[1]

[1]Department of Chemistry, Freshman Engineering Department, Prasad V. Potluri Siddhartha Institute of Technology, Kanuru, Vijayawada-520007, India
[2]Department of Chemistry, P.B. Siddhartha College of Arts and Science, Vijayawada-520010, India
[3]Division of Chemistry, Department of Sciences and Humanities, Vignan's Foundation for Science, Technology and Research, Vadlamudi, Guntur-522213, India

*Corresponding author: E-mail: pavani@pvpsiddhartha.ac.in

Received: 29 October 2023; *Accepted*: 8 December 2023; *Published online*: 31 January 2024; AJC-21519

A novel, streamlined and highly selective isocratic liquid chromatographic method has been developed for the quantification of impurities present in anagrelide. Exceptional resolution between anagrelide and its impurities was accomplished utilizing a Waters Nova Pack C18 column (250 mm × 4.6 mm, 4 µm), employing a phosphate buffer with a pH of 4.4 as mobile phase A and a blend of acetonitrile and methanol with buffer in a 30:40:30 v/v/v ratio as mobile phase B. The column oven was maintained at 35 ºC and the mobile phase flowed at a constant rate of 1.0 mL/min, while detection was set at 254 nm. Rigorous validation was conducted to assess accuracy, precision, specificity, linearity and sensitivity. Validation studies have unequivocally established the HPLC method's precision, specificity, rapidity, reliability and reproducibility. Linearity was ascertained for both anagrelide and its impurities, exceeding an R^2 value of 0.95. The limits of detection (LOD) and limit of quantitation (LOQ) were determined to be more than sufficient for precise estimation. This method was validated in accordance with the stringent ICH guidelines. The relative standard deviation (RSD) for intra-day and inter-day precision remained consistently below 5%. Percentage recovery demonstrated excellent agreement, affirming the simplicity, specificity, precision and enhanced accuracy of method for determining related substances in pharmaceutical substances and various anagrelide-containing dosage forms.

Keywords: Anagrelide, Related substances, Stability indicating, Liquid chromatography, Validation, Specificity.

INTRODUCTION

Anagrelide, chemically identified as 6,7-dichloro-1,5-dihydroimidazo[2,1-*b*]quinazolin-2(3*H*)-one (Fig. 1), is a potent pharmacological agent recognized for its capacity to reduce blood platelet levels significantly. This therapeutic compound is specifically indicated for the management of essential thrombocytosis [1] and its mechanism of action revolves around the inhibition of megakaryocyte maturation into platelets [2]. The hydrochloride form of anagrelide received approval from the U.S. Food and Drug Administration (FDA) in year 1997, marking a significant milestone in the treatment of patients afflicted with thrombocythemia stemming from myeloproliferative disorders. Its primary therapeutic objectives encompass the reduction of elevated platelet counts and mitigation of the associated risks of thrombosis, along with the amelioration of symptoms, including thrombo-hemorrhagic events. Notably, anagrelide, when administered at therapeutic doses, does not yield noteworthy alterations in white cell counts or coagulation parameters. Furthermore, its impact on red cell parameters, while existent, remains of minimal clinical significance. Mechanistically, anag-

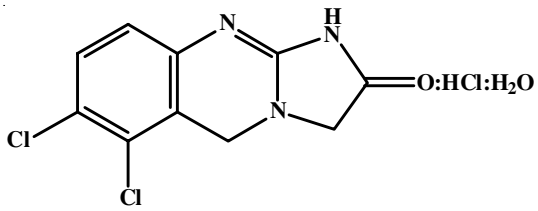

Fig. 1. Structure of anagrelide

relide, functions by inhibiting cyclic AMP phosphodiesterase III (PDEIII). PDEIII inhibitors have the ability to impact platelet aggregation, however, significant suppression of platelet aggregation becomes visible only at anagrelide dosages greater than those required for the primary goal of lowering platelet count [3,4].

The literature review revealed numerous chromatographic methods for the quantification of anagrelide in pharmaceutical formulations, with some focusing on anagrelide and its degradation products [2-4]. Moreover, several studies have delved into the challenging task of estimating anagrelide in combination with other drugs [5-7]. Additionally, the assessment of anagrelide and its metabolic profile in plasma samples has been undertaken through LC-MS and HPLC techniques [8-11]. In course of developing these analytical methods, two potential impurities, designated as impurity-4, were found within the drug product during stress degradation. Impurity-4 was found to be generated as a result of the dehydration of one mole of anagrelide in conjunction with one mole of tartaric acid. Tartaric acid is used as an excipient in the core pellets of formulation. Another impurity, known as impurity-G, is formed during the process of hydrolysis degradation of anagrelide, resulting in the replacement of the pyridine group with a hydroxyl group. It is imperative to acknowledge that the levels of tartaric acid-adduct impurity (impurity-F) and 4-hydroxy impurity (impurity-G) may increase over the course of the shelf-life of finished product. Therefore, diligent monitoring of these degradants becomes an indispensable and of paramount importance. In response to this requirement, a robust RP-LC method specifically intended for the separation and qunatification of all the seven identified impurities (Fig. 2) of anagrelide is developed. The proposed chromatographic proce-dure underwent comprehensive validation, encompassing the establishment of limits of detection (LOD) and quantification (LOQ), selectivity, precision, linearity, recovery and robustness.

In addition, the accuracy of the developed method was also confirmed by conducting placebo and drug product stress degradation studies, following the rules established by the International Council for Harmonization (ICH) [6]. Till date, no published method exists for the assessment of impurities in anagrelide capsules. This study presents an easy, simple and efficient method that could be useful for identifying anagrelide pollutants with high accuracy.

EXPERIMENTAL

The highest purity analytical grade chemicals *viz*. sodium hydroxide, potassium dihydrogen phosphate, orthophosphoric acid, hydrochloric acid, acetonitrile and methanol were procured from Merck India Ltd.

In the pursuit of method development and validation, the liquid chromatography systems employed were none other than the Waters Alliance 2695 separation module, in tandem with the 2998 diode array detector (DAD). The data generated by this setup were analyzed using the Waters Empower-2 software, Waters Corporation, MA, USA. For the chromatographic separation, a Waters Nova-Pak C18 column (250 mm × 4.6 mm, 4 µm) was selected. The unique characteristics and specifications of the column proved instrumental in achieving the desired separation of analytes.

Chromatographic conditions: The separation of related substances and impurities was accomplished through the utilization of a Waters Nova-Pak C18 column (250 mm × 4.6 mm, 4 µm) employing a gradient elution mode. This entailed the use of mobile phase A and mobile phase B, applied over a programmable 65 min timeframe. Mobile phase A was prepared by dissolving 1.36 g of potassium dihydrogen phosphate in 1000 mL of water with the solution pH adjusted to 4.4 using dilute orthophosphoric acid solution. In contrast, mobile phase B was a carefully degassed combination of mobile phase A, acetonitrile, and methanol in a precise proportion of 30:40:30 by volume. Elution was executed at a consistent flow rate of 1.0 mL/min, while maintaining the column oven at a stable temperature of 35 ºC. Wavelength monitoring occurred at 254 nm to track the elution of compounds. The injection volume was set at 20 µL, with a total run time of 65 min. As for the diluent, it consisted of a degassed blend of 0.01 N HCl and acetonitrile, harmoniously mixed in a ratio of 70:30 v/v. This solution was thoughtfully prepared to facilitate the analysis of samples.

Preparation of standard solution and control solution: The standard stock solution was prepared by dissolving 30 mg of anagrelide in a minimum amount of distilled water and then adjusted to volume by carefully adding the diluent in a 200 mL volumetric flask. In next step, 5 mL of the standard stock solution was diluted to 100 mL using the same precise diluent to prepare the working standard solution.

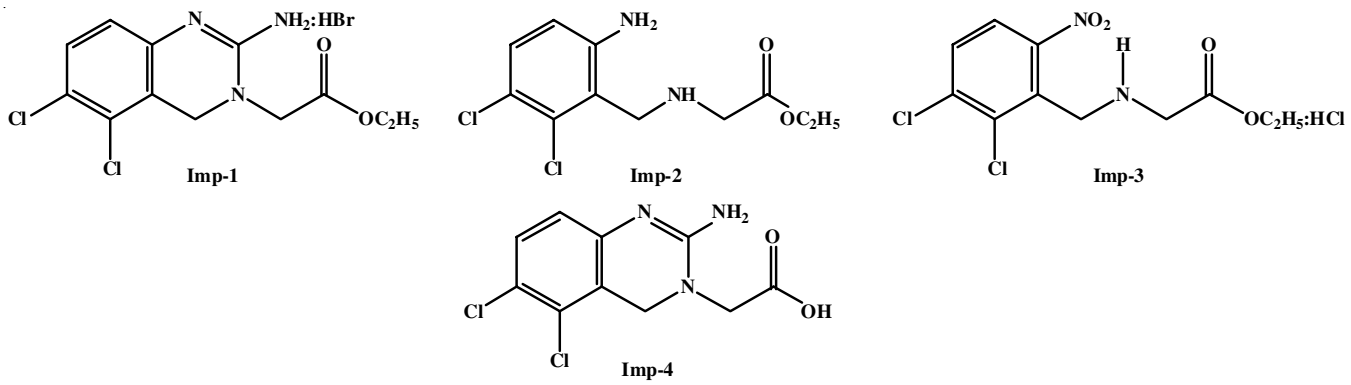

Fig. 2. Structure of anagrelide impurities

Preparation of test solution: A total of 10 commercially available capsules containing anagrelide were procured from the local market. These capsules were carefully crushed to a fine powder using a pestle and mortar equivalent to 100 mg of drug, transfer it to a 100 mL volumetric flask and after that the specified diluent was added till it reached the mark. Subsequently, a sonication process was initiated, subjecting the suspension to ultrasonic energy for a duration of 10-15 min to ensure thorough dissolution. After filtered through 0.45 µm membrane filters, the suspension was carefully injected into the LC system for analysis.

Method validation: The validation of this approach was carried out in accordance with the standards established by the International Conference on Harmonization (ICH) guidelines. The comprehensive validation process encompassed multiple critical aspects, including specificity, forced degradation studies, sensitivity, linearity, accuracy, precision, solution stability, mobile phase stability and robustness.

System suitability: The system suitability assessment was methodically conducted by subjecting the standard solution to six consecutive analyses. The rigorous evaluation involved the precise calculation of critical parameters, including the theoretical plates and tailing factor for the main peak as well as the signal-to-noise (S/N) ratio for the sensitivity solution.

Specificity/selectivity and forced degradation studies: Each individual impurity solution was prepared and subsequently injected into the chromatograph at a predefined specification level. This process was conducted in conjunction with the introduction of a spiked sample. The optimum chromatographic conditions were strictly followed with the diluent acting as blank. Similarly, a series of forced degradation experiments was conducted to demonstrate the intrinsic ability of the method to detect and quantify the contaminants under such conditions.

LOD and LOQ: In order to guarantee that the analytical results are absolutely accurate, the LOD and LOQ were determined as the lowest concentrations of the analyte peaks that could be detected and accurately measured. In order to guarantee that the analytical results are absolutely accurate, the LOD and LOQ were determined as the lowest concentrations of the analyte peaks that could be detected and accurately measured.

Precision and repeatability: Two separate aspects were carefully considered while evaluating the accuracy of the method: repeatability, which covered accuracy within a single day and intermediate precision, which included accuracy between days. The same analyst monitored the study of intra-day variability, which took place over the course of a single day. However, a separate analyst on different days was responsible for inter-day precision.

To establish the repeatability of the analytical method, a series of replicate injections (n = 6) of spiked samples was rigorously subjected to analysis. This comprehensive examination of precision extended to the measurement of retention times and peak areas for both anagrelide and its associated impurities.

Linearity: The linearity was carefully assessed using a systematic approach that included the preparation of spiked standard solutions containing contaminants at various concentration levels. These concentration levels encompassed six distinct points (n = 6), ranging from the limit of quantification (LOQ) to 150% of LOQ. Specifically, the concentrations evaluated were LOQ, 50%, 75%, 100%, 125% and 150% of LOQ. To appraise the linearity, the %RSD (relative standard deviation) and the *y*-intercept of the calibration curve, which represents the linear regression equation were also computed.

Accuracy: Recovery experiments were carried out in a systematic manner to determine the precision of impurity using the standard addition method. This study was conducted with utmost precision, involving triplicate analyses at multiple concentration levels, namely the limit of quantification (LOQ), 50%, 100% and 150% of the specified concentration. The resulting data were subjected to rigorous analysis and the percentage recoveries were accurately calculated. This procedure served as a critical measure to evaluate and ensure the precision and reliability of the method in quantifying impurities.

Solution stability and mobile phase stability: The solution stability of anagrelide and its related compounds was evaluated according to a strict methodology within the method framework. The process involved adding spiked sample solutions to volumetric flasks that were tightly sealed and then stored at room temperature (on a benchtop) for 48 h. Simultaneously, the stability of mobile phase was also thoroughly examined during the same 48 h period. For this, freshly prepared sample solutions were systematically injected at 24 h intervals. It is important to highlight that the composition of mobile phase exhibited stability and did not undergo any modifications during the entirety of this stability investigation.

Robustness: In order to evaluate the robustness of the analytical method that was developed, an intended deviation in the chromatographic conditions was carefully implemented and examined, in accordance with the system applicability criteria specified in the United States Pharmacopeia (USP). Within the framework of these robustness analyses, the determination of selectivity factors for all impurities, including anagrelide, occupied the utmost importance. The results obtained from this procedure were subsequently compared to those obtained from the standard experiment. The assessment of the mobile phase flow rate was an important factor in determining the robustness of the method. At first, a constant flow rate of 1.0 mL/min was chosen to examine the manner in which it affected the peak characteristics, with a specific emphasis on resolution and tailing between anagrelide and its related substances, in accordance with USP standards. Subsequently, the flow rate was carefully adjusted with 0.2 unit increases, spanning from 0.8 to 1.2 mL/min, in order to better understand its effects. Further investigation was conducted to fully examine the impact of column temperature, encompassing a temperature range of 30 ± 5 ºC, as an integral component of the comprehensive robustness assessment.

RESULTS AND DISCUSSION

The fundamental goal of this study is to provide a stability indicating chromatographic method, which is specifically designed for the successful separation of impurities present in anagrelide. The initial phase of method development was initiated

using an isocratic approach by employing a mobile phase comprising a pH 3 phosphate buffer and acetonitrile in a fixed ratio of 75:25. This was executed while utilizing a YMC Pack-C8 column and the flow rate was set at 1.0 mL/min. Unfortunately, this initial approach did not yield the desired separation of impurities from the main compound.

In light of this, several attempts were made to refine the method. However, given the persistent challenge of impurity separation under isocratic conditions, the approach was modified to a gradient elution mode. In this configuration, mobile phase A consisted of a pH 4.4 buffer, prepared by dissolving 1.36 g of potassium dihydrogen phosphate and adjusting the pH to 4.4 using dilute orthophosphoric acid. The composition of mobile phase B consisted of a mixture of buffer, acetonitrile, and methanol in a well-proportioned ratio of 30:40:30 v/v. By combining gradient elution with time-programmable elution, contaminants were successfully and clearly separated from the principal peak, with no undesired interference from the blank matrix.

Method validation

System suitability: The system suitability solution was meticulously prepared, following the same procedures outlined in the standard solution preparation. This rigorous process was carried out with precision to achieve a final concentration of anagrelide at 7.5 µg/mL as depicted in Fig. 3.

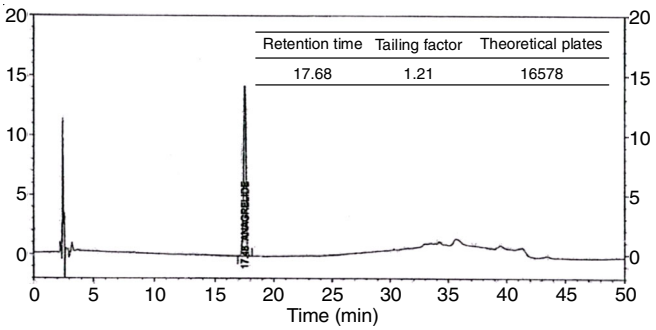

Fig. 3. Representative chromatogram of standard solution

Specificity and forced degradation: To affirm the specificity and its intrinsic quality as a stability indicating approach, a comprehensive set of analytical evaluations was undertaken. The analysis of individual impurities, spike samples and blank matrices through the utilization of the developed method (Figs. 4 and 5). Remarkably, during this rigorous evaluation, no ins-

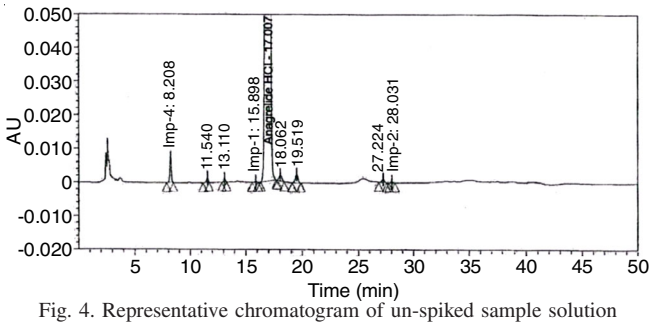

Fig. 4. Representative chromatogram of un-spiked sample solution

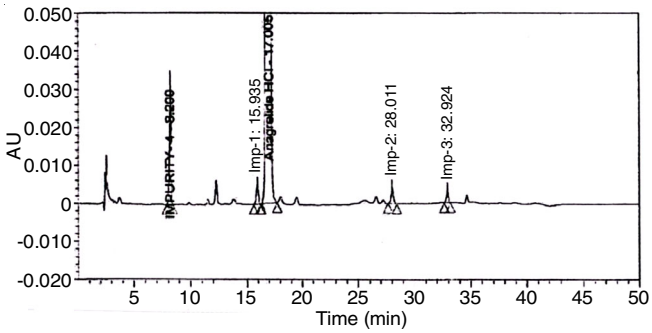

Fig. 5. Representative chromatogram of spiked sample solution

tances of blank interference were detected at the retention times corresponding to anagrelide and its related impurities. As the convincing results show the high selectivity and specificity, thus, this approach is ideal for the precise estimation of the impurities of anagrelide.

In addition, a series of forced degradation tests were conducted by following the standards set by the International Council for Harmonization (ICH) to determine the selectivity and stability indicating method. Various stress conditions, including acid hydrolysis, base hydrolysis, peroxide-induced degradation and thermal hydrolysis, were investigated. The evaluations of peak purity under all stress conditions proved that the approach is very selective and indicate the stability also (Table-1).

LOD and LOQ: The linearity approach was used methodically to determine the limit of detection (LOD) and limit of quantification (LOQ). The resulting values for LOD, LOQ, and the related precision at the LOQ level have been thoroughly obtained and the results are shown in Table-2.

System precision: Using six separate investigations, we determined the reference solution's peak area and % relative standard deviation for several anagrelide injections. Remarkably,

TABLE-1

Sample	Condition	Net degradation (%w/w)	Peak purity data of anagrelide	
			Purity angle	Purity threshold
As such	As per method	N/A	0.777	2.731
Acid degradation	10 mL. ACN + 0.1 N HCl (50:50 v/v) and 4 mL, 2 N HCl, heated on mantle for 60 min @60 °C neutralized with 2 N NaOH	3.40	0.340	2.481
Alkali degradation	8 mL 0.1 N HCl + ACN (70:30 v/v), heated on mantle for 60 min @60 °C and final volume made with 0.2 N HCl + ACN (70:30 v/v)	3.41	0.274	1.259
Thermal degradation	1 h@60 °C	3.88	0.447	2.025
Peroxide degradation	5 mL of 10% H_2O_2 heated on mantle for 60 min @60 °C final volume made up volume with 0.2 HCl, ACN (70:30 v/v)	7.15	0.289	1.016

TABLE-2
LOD AND LOQ VALUES AND PRECISION AT LOQ

Parameter	Imp-1	Imp-2	Imp-3	Imp-4	Anagrelide
LOD (µg/mL)	0.02	0.05	0.03	0.05	0.02
LOQ (µg/mL)	0.07	0.18	0.11	0.17	0.06
Precision at LOQ (%RSD)	1.9	6.4	2.7	3.1	2.8

anagrelide has a relative standard deviation (RSD) of less than 10% (Table-3). Confirming the remarkable precision of the analytical system, the result is fully consistent with the previously established the acceptance criteria.

TABLE-3
SUMMARY OF PEAK AREAS OF THE ANAGRELIDE STANDARD

Injection No	Anagrelide	Injection No	Anagrelide
1	210245	5	226478
2	226458	6	214578
3	209785	Mean area	217023
4	214597	%RSD	3.50

Method precision: The analytical analysis results (Table-4) of a sample containing anagrelide solution selectively spiked with contaminants at a concentration equal to 100% of the pre-established specification limit rigorously confirmed the method's accuracy.

TABLE-4
SUMMARY OF RESULTS FOR PRECISION OF THE METHOD

Injection No.	IMP-1 (%)	IMP-2 (%)	IMP-3 (%)	IMP-4 (%)
Mean (%)	92.5	98.6	101.7	105.4
RSD (%)	1.5	0.8	0.4	1.9

Linearity: In order to evaluate the linearity of the HPLC method, standard solutions containing anagrelide and its impurities were injected simultaneously. From 0.12 µg/mL to 3.75 µg/mL, the concentration levels for anagrelide and its impurities 1 through 4 ranged from the limit of quantification (LOQ) to an impressive 300%. In this analysis, the peak area responses of anagrelide and its impurities were carefully plotted against their corresponding concentrations. After that least squares regression approach was conducted a thorough linear regression study. Results from the calibration procedure showed that anagrelide and its impurities had calibration curves with correlation values higher than the threshold value of 0.99.

Accuracy: To ensure precise impurity quantification, the spiked samples were carefully prepared and added them to the drug ingredient at concentrations, which are exactly equal to or more than the desired limits (Fig. 6). Consistently, the percentage recovery values obtained for each impurity were within the specified range of about 85% to 115%, fitting closely with the predetermined standards. Surprisingly, the relative standard deviation (RSD) values for the recoveries related to all the contaminants considerably boosted the accuracy evaluation (Table-5). The low RSD results (> 2%) demonstrated the analytical method's exceptional accuracy and dependability.

Additionally, the method's solution stability and robustness were thoroughly tested and the results clearly showed

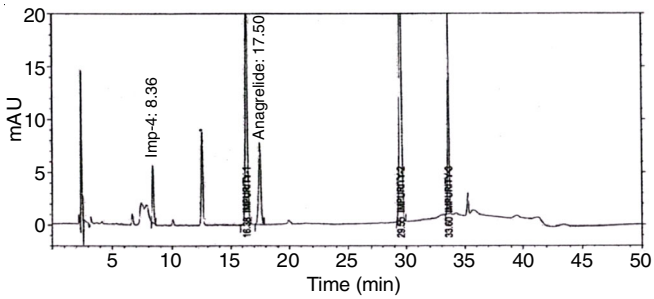

Fig. 6. Representative chromatogram of accuracy solution at 100% level

TABLE-5
SUMMARY OF RESULTS FOR RECOVERY/ ACCURACY OF THE METHOD AT 100%LEVEL

Impurity	Level	Amount (%w/w)		Recovery (%)
		Added	Found	
IMP-1	100%	0.427	0.407	88.8
IMP-2	100%	0.520	0.470	98.7
IMP-3	100%	0.446	0.446	92.5
IMP-4	100%	0.677	0.507	105.8

that the developed approach meets all the requirements according to the ICH guidelines. Thus, the evaluation proves that the procedure is prepared for reliable and acceptable analytical use.

Conclusion

A novel analytical method, characterized by its simplicity, sensitivity and robustness has been developed for the precise estimation of related substances present in anagrelide. This method stands as a versatile tool, amenable to the quantification of impurities at various stages of drug substance production, encompassing both the drug substance itself and its diverse dosage forms. The validation of the method has been conducted with the standards to the ICH guidelines, affirming its exceptional specificity and reliability. The proposed method perfectly matches with the requirements established by several regulatory recommendations, making it a reliable and necessary method for assessing anagrelide impurities in bulk pharmaceuticals and pharmaceutical dosage forms.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

REFERENCES

1. D.A. Hussar, *J. Am. Pharm. Assoc.*, **38**, 155 (1998); https://doi.org/10.1016/S1086-5802(16)30315-1
2. A. Tomer, *Blood*, **99**, 1602 (2002); https://doi.org/10.1182/blood.v99.5.1602
3. A.I. Schafer, *N. Engl. J. Med.*, **350**, 1211 (2004); https://doi.org/10.1056/NEJMra035363

4. G. Birgegard, M. Bjorkholm, J. Kutti, G. Larfars, E. Lofvenberg, B. Markeva, M. Merup, J. Palmblad, N. Mauritzson, J. Westin and J. Samuelsson, *Haematologica*, **89**, 520 (2004); http://www.ncbi.nlm.nih.gov/pubmed/15136214
5. E.H. Kerns, J.W. Russell and D.G. Gallo, *J. Chromatogr., Biomed. Appl.*, **416**, 357 (1987); https://doi.org/10.1016/0378-4347(87)80520-0
6. Z. Zhu, R. Gonthier and L. Neirinck, *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.*, **822**, 238 (2005); https://doi.org/10.1016/j.jchromb.2005.06.021
7. Guidance for Industry, ICH Q2B. Validation of Analytical Procedures: Methodology, Food and Drug Administration; Rockville, MD, USA (1997).
8. ICH, Q2 (R1), Validation of Analytical Procedures: Text and Methodology (1996).
9. Draft Revised Guidance on Impurities in New Drug Substances, Q3A (R), Federal Register; International Conference on Harmonization, pp. 45085-45090 (2000).
10. J. Voglova, V. Maisnar, M. Beránek and L. Chrobak, *Vnitr. Lek.*, **52**, 819 (2006).
11. P.E. Petrides, *Semin. Thromb. Hemost.*, **32**, 399 (2006); https://doi.org/10.1055/s-2006-942760