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THERE ARE NO PERFECT ANALYTICAL METHODS - OPTIMIZATION STUDY OF A PHARMACOPOEIA METHOD FOR CLOPIDOGREL ANALYSIS

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ABSTRACT. Clopidogrel is a chiral compound, (+)-(S)-enantiomer has antiplatelet activity, while (-)-(R)-clopidogrel is considered an impurity of the active substance. The development of HPLC methods for optical purity analysis involves the use of expensive chiral columns and the methodologies could be different in the two well recognized compendia, European (Ph.Eur.) and United Stated (USP) Pharmacopoeias. The aim of this study was to optimise the chiral chromatographic assay of clopidogrel and its impurities from tablets taking into consideration the official pharmacopoeia provision by testing the influence of the variation of different chromatographic parameters on the relative retention times of clopidogrel and official impurities in order to achive the separation performance parameters described in USP43. HPLC-UV enantioseparation of clopidogrel was performed on a column with ovomucoid as a stationary phase chiral selector and phosphate – acetonitrile mixtures as mobile phase. The official relative retention of clopidogrel and its A and C impurities could not be achieved in this study, neither by applying a classical optimization methodology nor by using the experimental design methodology which raises the issue of reviewing the pharmacopoeia provisions. A comparative discussion between the two different approaches regarding chiral separation of clopidogrel in Ph.Eur. and USP is provided.

Keywords: *clopidogrel, chiral separation, HPLC, ovomucoid, enantioseparation.*

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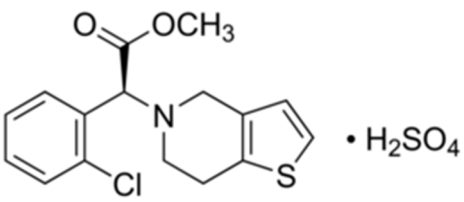
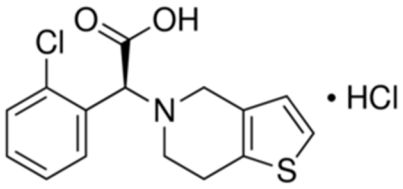
INTRODUCTION

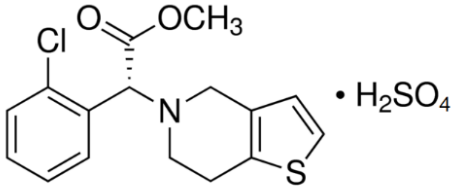
Clopidogrel (CLPD) is an antiplatelet agent, an inhibitor of P2Y12, commonly used in the secondary prevention of cardiovascular disease including transient ischemic attack, stroke or peripheral artery disease [1]. Although modern P2Y12 inhibitors such as prasugrel and ticagrelor are marketed, CLPD still has financial advantages over them and unlike aspirin, clopidogrel has the advantage of being used in patients at high risk of bleeding [2]. CLPD is a prodrug transformed into its active metabolites (2-oxo-CLPD and CLPD thiol) mainly via CYP2C19, but also with the participation of other enzyme systems CYP1A2, CYP2B6 and CYP3A, its active metabolites bind irreversibly to the receptor P2Y12 in blood platelets, preventing their aggregation [3].



Clopidogrel bisulphate



Clopidogrel Impurity A



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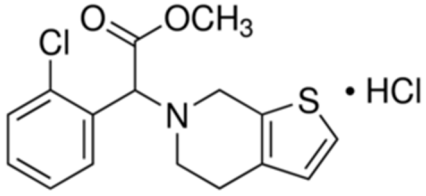

Clopidogrel Impurity B

Figure 1. Chemical structures of CLPD and chemically related impurities specified in pharmacopoeias (USP, Ph. Eur. 10.5)

CLPD contains an asymmetric carbon atom and presents optical isomerism, but only the (+)-(S) enantiomer has antiplatelet activity, while the (-)-(R) enantiomer lacks this pharmacological effect. Additionally, in animal studies, it was observed that the (-)-(R) enantiomer triggered seizures at high doses [4,5], therefore, it is considered an impurity of (+)-(S)-CLPD [6].

Considering the chirality of CLPD, the possibility of enantiomerism, as well as the activation of the compound *in vivo*, a special interest is given to its impurities, including those from the active substance synthesis or other classes of compounds resulting from degradation processes which are described in the literature as well [7,8]. According to monographs from United States Pharmacopoeia 43st Edition (USP43) and the European Pharmacopoeia 10.5 (Ph. Eur. 10.5), the impurities of interest are: carboxylic acid form impurity A (IMP A), positional stereo isomer impurity B (IMP B) and a chiral isomer of clopidogrel impurity C (IMP C) (Figure 1).

Pharmaceutical impurities are compounds present in the final product that are nor drug not excipient. Impurity profiling is a condition s*ine qua non* for the quality of a substance and also for the registration process as imposed by regulatory authorities, including the Food and Drug Administration (FDA) or European Medicines Agency (EMA), and according to pharmacopoeia provisions such as United States Pharmacopoeia (USP) or European Pharmacopoeia (Ph. Eur.) [9-11].

Considering the tests imposed by regulating authorities to pharmaceutical manufacturers in terms of quality and quantity of active substance in raw material or pharmaceutical forms, the relatively large number of samples analyzed but also the costs imposed by these analyzes (expensive and short-lifespan chiral columns in the analysis of impurities, reducing the volume of solvents that are not environmentally friendly or adaptation of the HPLC systems to specific working conditions), the methods described in pharmacopoeias are in general well optimized.

Our study aims to combine the classical approach by applying the USP43 HPLC method for the determination of CLPD and its impurities and Quality by Design (QbD) method in order to achieve the suitability criterion regarding the relative retention by respecting the accepted limits of variation for different chromatographic parameters and, nevertheless, to find out how appropriate is the optimization under these regulations in the case of CLPD.

RESULTS AND DISCUSSIONS

Influence of several chromatographic parameters on the efficiency and selectivity of CLPD determination

First, the chromatographic conditions described by the USP43 method for the determination of CLPD in raw material and tablets were reproduced: ovomucoid column (150x4.6 mm, 5 μm), mobile phase composition 75% phosphate 10 mM, 25% ACN, flow 1 mL/min, column temperature 25 °C,

wavelength 220 nm, injection volume 10 μL. In these chromatographic conditions IMP A, CLPD and IMP C had the unadjusted relative retention (RRT) (retention time ratio) of 0.683, 1 and 1.504, respectively (Figure 2).

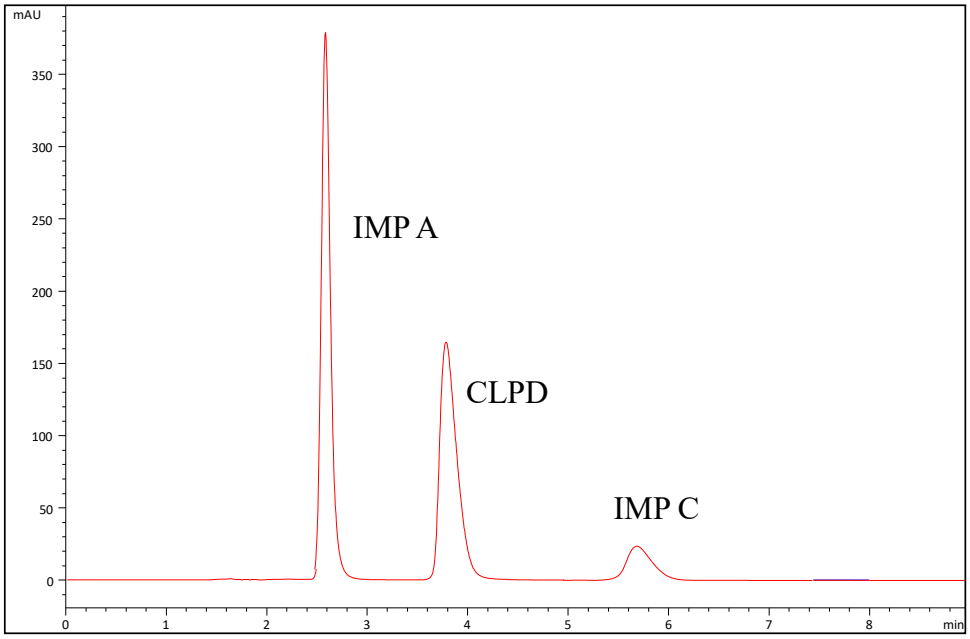

Figure 2. Chromatogram of a mixture consisted of IMP A (100 μg/mL) R_t = 2.586 min, CLPD (100 μg/mL) R_t = 3.785 min and IMP C R_t = 5.695 min (unknown concentration) in the mobile phase. Chromatographic conditions as stated in USP43 clopidogrel tablets monography

According to the official USP43 monography of CLPD tablets, RRT should be 0.5 (IMP A), 1 (CLPD) and 2 (IMP C), respectively. Consequently, changes in chromatographic conditions were performed but in respect of the limits imposed by pharmacopoeia (see Table 4), however other chromatographic conditions were modified to larger limits, such as phosphate salt concentration. Changes in experimental chromatographic conditions produced minor changes in the retention time of IMP A, therefore it was tested whether a drastic change in phosphate concentration of aqueous mobile phase to values of twice and half from the official limit in the mobile would modify the capacity factor of this substance to obtain the RRT = 0.5.

In order to obtain a RRT = 0.5 for IMP A, first, an attempt was made to obtain an appropriate mixture between aqueous component (10 mM potassium dihydrogen phosphate) of the mobile phase and the organic

component. Different conditions were tested on a standard binary mixture of IMP A and CLPD. As seen in Figure 3.a, at 25^0C, an increased percent of phosphate increases the resolution of the peaks, with a consecutive decrease in the symmetry T of the CLPD peak, while the symmetry T of IMP A peak does not seem to be influenced (Table 1).

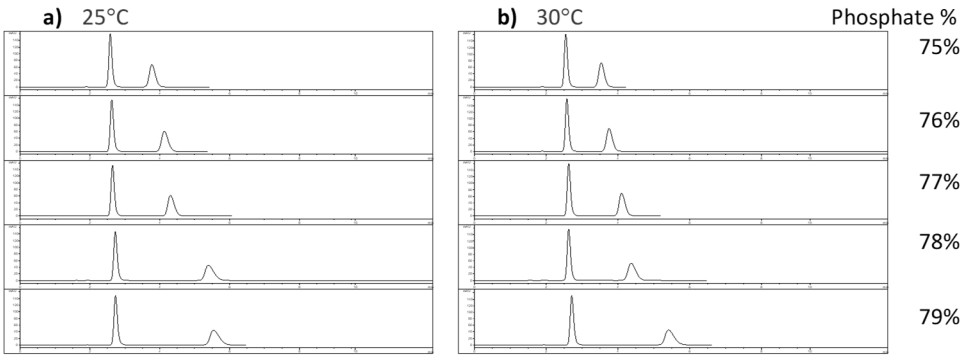

Figure 3. The influence of 10 mM phosphate percent (75%, 76%, 77%, 78% and 79%) in the mobile phase on the separation of IMP A and CLPD (standard aqueous mixture of CLPD and IMP A, 100 µg/mL each analyte) at a) 25°C and b) 30°C. The other chromatographic conditions correspond to USP43 provisions.

Increasing the temperature of the chromatographic column to 30°C did not improve the symmetry of the peaks when using similar compositions of the mobile phase (Figure 3.b and Table 2). The RRT = 0.5 for IMP A was obtained for a percentage in the mobile phase of 78% phosphate at 25°C and a percentage of 79% at 30°C.

Table 1. The influence of phosphate percent in the mobile phase and the column temperature on the separation and symmetry of IMP A and CLPD peaks. The other chromatographic parameters were set according to USP43 provisions

Phosphate 10 mM (V/V)*	**25 °C**			**30 °C**		
	RRT IMPA	**$T_{IMP\ A}$**	**T_{CLPD}**	**RRT IMP A**	**$T_{IMP\ A}$**	**T_{CLPD}**
75%	0.684	0.763	0.746	0.720	0.758	0.768
76%	0.635	0.761	0.720	0.690	0.760	0.742
77%	0.614	0.762	0.703	0.640	0.760	0.716
78%	0.505	0.764	0.627	0.600	0.756	0.672
79%	0.492	0.762	0.616	0.501	0.764	0.622

** isocratic conditions, ACN up to 100% (V/V) in the composition of the mobile phase*

The influence of flow rate and decreasing of column temperature on the separation of IMP A and CLPD was tested by maintaining the other chromatographic parameters as specified in USP43. Two tests were performed at 20°C, one at a flow rate of 1 mL/min where RRT IMP A = 0.67 min and the second, at 1.5 mL/min where RRT IMP A = 0.652 min, which indicates that the 50% flow rate increases and a lower temperature of separation with 5 units do not significantly influence the RRT of IMP A in comparison with the results obtained in USP43 conditions.

In terms of absolute retention time, IMP A is not significantly influenced by modifying the proportion between the aqueous and organic phase, by a change with 5°C of the column temperature, or by a 50% increase in mobile phase flow. The observed RRT variations were due to the sensitivity of CLPD retention to these chromatographic condition changes.

Taking into consideration the previous results, univariate or multivariate modifications of the reference chromatographic conditions in the USP43 were made, using a three component mixture of CLPD, IMP A and IMP C, respectively. The results are presented cumulatively in Table 2. In addition to varying the chromatographic conditions between the limits accepted by USP43, a double and a half concentration level of phosphate in the mobile phase, and the replacement of a part of ACN with MeOH were also tested. The chromatographic conditions modifications take into consideration the stability of ovomucoid column, very sensitive to degradation at high organic content in mobile phase.

Table 2. The RRT of IMP A and IMP C by uni- or multivariate changes of the reference USP43 chromatographic conditions

Mobile phase - phosphate, % (V/V)			**Mobile phase - ACN, % (V/V)**	**Mobile phase – MeOH, % (V/V)**	**Flow, mL / min**	**t, °C**	**RRT**	
10 mM	**20 mM**	**5 mM**					**IMP A**	**IMP C**
75	0	0	25	0	1	25	0.684	1.485
0	75	0	25	0	1	25	0.506	1.510
0	0	75	25	0	1	25	0.753	1.477
82.5	0	0	17.5	0	1	25	0.302	2.131
0	75	0	20	5	1	25	0.514	1.693
80	0	0	20	0	1	30	0.421	1.896
80	0	0	20	0	1.5	35	0.510	1.586
75	0	0	25	0	1	20	0.670	1.590
75	0	0	25	0	1.5	20	0.652	1.630

Despite these adjustments, the relative retention times as according to the reference and, in the same time, an acceptable symmetry for CLPD and IMP C could not be achieved; higher percent of phosphate in the mobile phase A, although lead to RRT values close to reference, caused an improper tailing phenomenon of CLPD and IMP C peaks (Figure 4).

Absolute retention times of IMP A vary in a range of 0.6 min under the selected conditions, while for CLPD and IMP C changes of up to 15 min (for IMP C) were observed. For IMP A, the capacity factor k' and the retention on the column were little influenced by the experimental conditions. For CLPD and IMP C, improving the selectivity leads to a decrease in the separation efficiency under the tested conditions (asymmetric and wide peaks).

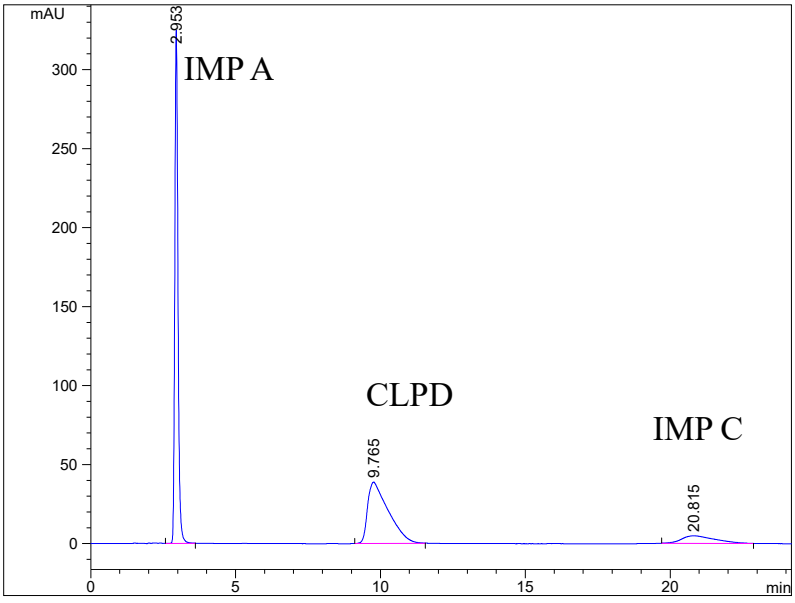

Figure 4. Chromatogram of a standard mixture of IMP A, CLPD and IMP C at 25°C, with a mobile phase consisting in 82.5% phosphate, 17.5% ACN, 1 ml/min, 220 nm

Increasing the percentage and concentration of phosphate leads to increased selectivity. Increasing the temperature reduces the separation between IMP A and CLPD, and between CLPD and IMP C, respectively; lowering the temperature has the opposite effect. Replacing a part of the ACN in the mobile phase with MeOH increases the separation between peaks, as it was accepted.

Optimizing chromatographic conditions through experimental design

All the experimental results obtained by varying different chromatographic parameters were used in an experimental design software Modde verse 12 to optimize the method with the main objective achieving the relative retention times for IMP A and C as mentioned in USP43. Calculations were performed and the proposed conditions are shown in Figure 5. The chromatogram obtained in these conditions is presented in Figure 6.

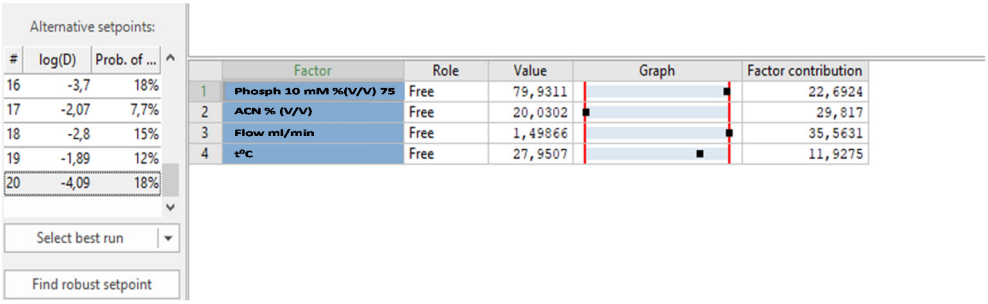

Figure 5. Optimization of the chromatographic conditions by experimental design

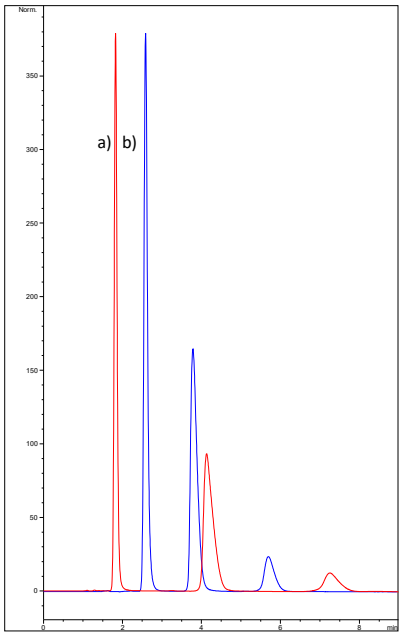

Figure 6. a) Experimental design conditions; b) USP43 conditions.

The absolute retention times were: $t_{R\ IMP\ A}$ = 1.826 min, $t_{R\ CLPD}$ = 4.13 min, $t_{R\ IMP\ C}$ = 7.253 min, and the relative retention RRT IMP A = 0.442, RRT CLPD = 1 and RRT IMP C = 1.756, respectively.

The comparative analysis of the two chromatograms, a decrease of the peak symmetry of IMP C was observed in the proposed conditions compared to USP43 method. The results were not satisfactory, probably due to the limited number of experimental input data.

Estimating the CLPD and IMP A content in pharmaceutical products

The USP43 method for CLPD determination was tested for the analysis of the active substance and IMP A from two types of tablets (sample 1 and 2) available on the Romanian market, as IMP A was the only impurity available in measurable quantities.

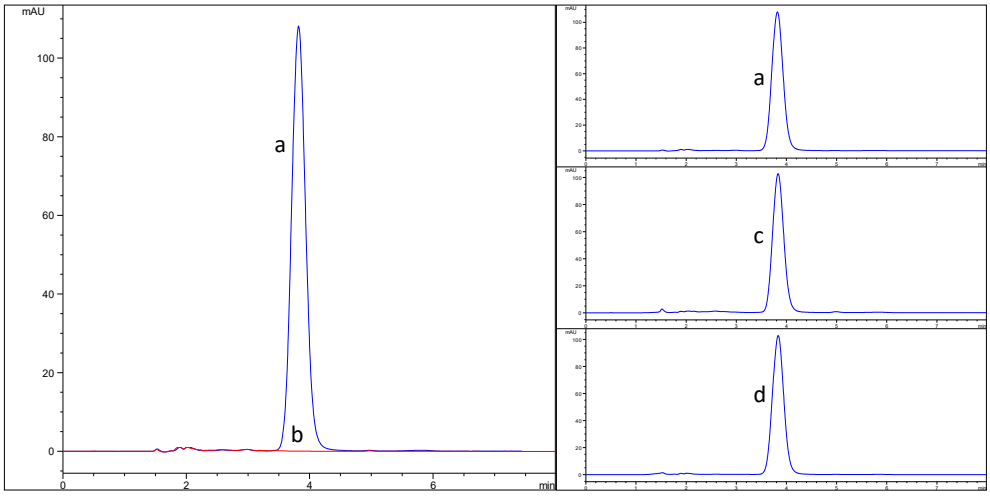

Figure 7. Chromatograms: a) CLPD standard solution (100 μg/mL in MeOH); b) Mobile phase; c) Sample 1 solution; d) Sample 2 solution. Chromatographic conditions: 75% phosphate 10 mM, 25% ACN, 1 mL/min, 25°C, 220 nm, injected volume 10 μL

The results obtained after the analysis of the two commercial tablets (containing 75 mg CLPD bisulphate) under official conditions of USP43 are presented in Table 3.

Table 3. The estimated content of active pharmaceutical ingredient and IMPA A of two commercial CLPD tablets

	CLPD, mg/tablet	Limits of acceptance USP43, mg/tablet	IMP A, %	IMP A limit of acceptance, USP43
Sample 1	71.89	67.5 ÷ 82.5	0.042	1.2%
Sample 2	73.29		0.013	

As it can be seen, the tablets follow the regulations in terms of active pharmaceutical ingredient and IMP A content. The experiment provides an estimate as method was not validated, but demonstrates on one hand the applicability of method itself and on the other hand the validity of the approach in the respective laboratory.

Comparison of different approaches of chiral HPLC resolution for clopidogrel in the context of official provisions

The United States Pharmacopoeial Convention (USP Convention) meets at 5-year intervals to add monographs to new substances introduced in therapy or to review existing monographs, usually due to advances in analytical techniques based on the recommendations of the Council of Experts. [12].

The most frequently used methods in the analysis of active substance and impurities in raw materials or pharmaceutical forms in the case of chiral molecules such as CLPD are high-performance liquid chromatography (HPLC) and ultrahigh-performance liquid chromatography (UPLC) with UV detection, and involve the use of chiral stationary phases (carbohydrate-based columns - amylose and cellulose derivatives, protein-based columns - α1-acid glycoprotein (AGP), albumin or vancomycin etc) [13-15], while for determinations of CLPD from biological samples HPLC coupled with a mass spectrometer (HPLC-MS) technique is used [16-18]. In the monographs regarding CLPD from Ph. Eur. 10.1 cellulose derivatives of silica are stipulated for chiral liquid chromatography tests, while the USP43 states the use of a protein derivatized silica, i.e. ovomucoid, for the assay of CLPD bisulfate and the tablets containing the same salt, and also for the determination of the related compounds in CLPD tablets [10,11]. The latter compendium also requires a polysaccharide column (a cellulose derivatized silica) for the determination of R enantiomer of CLPD. In the Japanese Pharmacopoeia (JPh17) a cellulose chiral column is stipulated for the determination of the optical isomer from the drug substance but an ovomucoid column is required when analyzing CLPD tablets [19].

In the case of determinations of compounds such as CLPD, the use of chiral columns involves high costs, therefore optimizing the methods of analysis especially in case of injection of high volumes of samples to detect low concentrations of chemically related impurities that can lead to premature destruction of a chromatographic column is extremely important. In literature there are many cases in which changes of analytical methods have been proposed that would bring improvements such as shortening the analysis time or prolonging the life of the column by using “milder” solvents, temperature applied to the column, mobile phase flow etc. For example, determination of somatropin through isoelectric focusing was changed with capillary zone electrophoresis in the January 2006 European Pharmacopoeia Supplement 5.3, because of a study elaborated by several laboratories [20].

Another example, the latest edition of Ph. Eur. uses heptane and ethanol, a mobile phase typical for normal phase chromatography to determine CLPD, which would imply the need to purchase an HPLC system equipped with tubing resistant to these solvents. Moreover, these solvents are toxic to the environment, while the USP method involves the use of more environmentally friendly solvents. In addition, in the case of the USP method of CLPD, the analysis time is shorter (under 10 minutes compared to 25 minutes in Ph. Eur. 10.1), but the column costs involved are higher [10,11].

In British Pharmacopoeia 2016 edition, in the CLPD Hydrogen Sulfate monograph it is recommended for the analysis of CLPD impurities by reversed-phase HPLC the use of a stationary phase end-capped octadecylsilyl silica gel type, and as mobile phase a mixture of MeOH and 0.96 g/L sodium pentanesulfonate monohydrate solution adjusted to pH 2.5 with phosphoric acid, ACN and MeOH, a flow rate of 1 mL/min and 30°C column temperature. To test the presence of chiral impurities, HPLC analysis is recommended on a silica gel column and a mobile phase containing ethanol and heptane, similar to the method in Ph. Eur. 10.1 [21].

The concept of QbD was introduced in 2004 by the U.S. Food and Drug Administration (FDA) and approved in 2005 by the International Conference on Harmonization (ICH) refers primarily to analytical methods for controlling the quantity and quality of the active substance. QbD is mainly applied to liquid chromatography analyzes (HPLC or UHPLC) usually in reversed phase and with UV detection to determine the active substance and impurities [22]. This concept can remove the influence that different variables can have on the results of the chromatographic analysis [23]. In the present study, the optimizing of USP43 method for determining CLPD, the QbD method produced results similar to those obtained in the laboratory in terms of modifying the main chromatographic parameters to obtain an efficient separation.

Limitations of the described experimental study

The limitations could be summarised as follows:

- Failed to work with all three official USP43 impurities;
- The method was not validated;
- The classical variation of the experimental conditions, within the limits provided by the compendial norms, did not allow the achievement of the separation conditions provided by the USP43 monograph;
- The aim was only to optimize the selectivity of the method, expressed by relative retention times, without taking into account the symmetry of the peak (which varies significantly on the ovomucoid column with the variation of chromatographic parameters).

CONCLUSIONS

HPLC analysis with chiral stationary phases is a common approach in pharmacopoeias, especially for enantiomeric purity tests, having major importance for substances used in therapy as a pure optical isomer.

The present study achieved relative retention times of CLPD and its IMP A and C only close to those specified in USP43, both by applying a classical optimization methodology and by using experimental design, which indicates the need of a possible revision of the official CLPD determination conditions.

EXPERIMENTAL SECTION

Chemicals and reagents

CLPD bisulfate, IMP A and IMP C were USP standards. HPLC-grade acetonitrile (ACN) and methanol (MeOH) were purchased from VWR Chemicals and J.T. Baker, respectively, and were used as solvents for standard solutions preparation. Pure potassium dihydrogen phosphate was purchased from Merck, Germany and was used for the preparation of the HPLC mobile phase. Distilled, deionized water was produced by a Direct-Q5 Millipore (Millipore SA, France) water system.

Chromatographic conditions

The HPLC stereoselective method of CLPD was performed on an Agilent 1100 Series System (Agilent Technologies, USA) equipped with a UV detector. As a chiral stationary phase, an Ultron OVM was used (150x4.6 mm

column, 5 µm particles, Shinwa Chemical/Agilent Technologies). The chiral selector consisted of ovomucoid glycoprotein immobilized on an aminopropyl silane-derivatized silica column. Different mobile phases were tested, containing different proportions of the aqueous component as phosphate buffer and various proportions of organic modifiers (methanol and acetonitrile). Concentrations of 5 mM, 10 mM, and 20 mM potassium dihydrogen phosphate in ultrapure water were tested as an aqueous mobile phase.

Samples, stock and standard solutions preparation

Stock solutions of 1000 µg/mL CLPD and IMP A in MeOH were prepared by weighting appropriate amounts with the aid of an AB 54 S Metter-Toledo, Switzerland, analytical balance, both solutions were then diluted to a concentration level of 100 µg/mL with MeOH. A stock solution with unknown concentration of IMP C was prepared by same method, as the small amount of standard available could not be weighed. A mixture of CLPD (100 µg/mL), IMP A (100 µg/mL) and IMP C was prepared with ultrapure water. Another standard mixture of CLPD and IMP A in MeOH was prepared as specified in USP43; the solution obtained had concentration levels of 40 µg/mL CLPD and 250 µg/mL IMP A. The latter mixture was diluted with the mobile phase according to the monograph and concentration levels of 1 µg/mL for CLPD and 6.25 µg/mL for IMP A were obtained.

CLPD tablets from two different producers, available on the Romanian market, were purchased and samples were prepared according to the USP43 monograph by weighing and grinding 20 tablets. A quantity of the ground tablet powder, corresponding to the average weight of one tablet, was suspended in 50 mL of MeOH using ultrasonic baths T700/H (Elma, Switzerland). The solution obtained was then diluted as specified and filtered through a syringe filter with proper porosity.

Reference chromatographic conditions (USP43):

- Ultron OVM column 150x4.6 mm, 5 µm (Agilent Technologies);
- Column temperature: 25°C;
- Mobile phase consisting of a mixture of ACN and potassium dihydrogen phosphate 10 mM;
- Flow rate: 1 mL/min;
- Sample temperature: 20°C;
- Injection volume: 10 µL;
- Detection: 220 nm.

Optimization of chromatographic conditions

The changes applied to the chromatographic conditions are limited and must vary between specific ranges according to Ph. Eur. 10.1 [10] and USP43 [11] (see Table 4).

Table 4. Adjustments that can be applied to the isocratic elution in HPLC analysis as stated by USP43 and Ph. Eur. 10

Variable	USP43	Ph. Eur. 10
Composition of the mobile phase, the minor component	±30% relative percent or ±10% absolute values (the widest range is chosen)	±30% relative percent or ±2% absolute values (the widest range is chosen)
pH of the aqueous phase component	±0.2 pH	±0.2 pH (or ±1.0 pH in analysis of non-ionizable substances)
Salt concentration in the buffer mobile phase component	±10%	±10%
Mobile phase flow rate	±50%; (higher limit is acceptable when changing the column size)	±50%; (higher limit is acceptable when changing the column size)
Column temperature	±10 °C	±10 °C

Experimental data acquisition and processing

Chromatographic data were obtained by the HPLC system software: Chemstation RevA 10.02[1757]. Optimization of the analytical method through experimental design was performed on a Modde vers 12 software.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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