Article

Determination of Genotoxic Azide Impurity in Cilostazol API by Ion Chromatography with Matrix Elimination

Boglárka Páll ¹, Zsuzsa Gyenge ¹, Róbert Kormány ¹ and Krisztián Horváth ^{2,*}

¹ Drug Substance Analytical Development Division, Egis Pharmaceuticals Plc., Keresztúri út 30-38, H-1106 Budapest, Hungary; pall.boglarka@egis.hu (B.P.); gyenge.zsuzsa@egis.hu (Z.G.); kormany.robert@egis.hu (R.K.)

² Research Group of Analytical Chemistry, University of Pannonia, Egyetem utca 10, H-8200 Veszprém, Hungary

* Correspondence: raksi@almos.uni-pannon.hu

Abstract: Cilostazol is a commonly used active pharmaceutical ingredient (API) to treat and reduce the symptoms of intermittent claudication in peripheral vascular disease. Recently, it was found to be a potential medicine in the effective treatment of COVID-19. In addition to the positive effects of this API, genotoxic sodium azide is used in the synthesis of cilostazol that can appear in the API. In this work, a method was developed for the determination of sodium azide (as azide anion) in cilostazol API at 7.5 ppm limit level by using ion chromatography (IC) and liquid–liquid extraction (LLE) sample preparation. The liquid–liquid extraction allows the application of high sample concentrations. Because of the low limit concentration (7.5 ppm), 500 mg sample was dissolved in 5 mL solvent. By using LLE for sample preparation, the huge amount of cilostazol was omitted and column overload was avoided. The developed method was validated in accordance with the relevant guidelines. Specificity, accuracy, precision, limit of detection and limit of quantification parameters were evaluated. The calculated limit of detection was 0.52 ppm (S/N:3) and the limit of quantification was 1.73 ppm (S/N:10) for sodium azide. The recovery of the sodium azide was 102.4% and the prepared solutions were stable in the sample holder for 24 h.

Keywords: azide; cilostazol; COVID-19; genotoxic impurity; validation; LLE

Citation: Páll, B.; Gyenge, Z.; Kormány, R.; Horváth, K. Determination of Genotoxic Azide Impurity in Cilostazol API by Ion Chromatography with Matrix Elimination. *Separations* **2021**, *8*, 162. <https://doi.org/10.3390/separations8100162>

Academic Editors: Alberto Cavazzini and Caroline West

Received: 28 July 2021

Accepted: 13 September 2021

Published: 23 September 2021

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Copyright: © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

The cilostazol is a platelet-aggregation inhibitor and arterial vasodilator, its long-term use may prevent stroke [1]. Recently, a network-based ranking was used to prioritize drugs to treat COVID-19 symptoms [2]. Cilostazol was the fourth on this list. Several studies were published about cilostazol long-term treatment safety. It was found by W.R. Hiatt et al. that the mortality was not higher in the treatment group, than the placebo group during the examined 42 months [3]. In addition to the long-term treatment safety of cilostazol, attention should be drawn to the possible impurities because these can cause deteriorate side effect for patients in the long term. Figure 1 shows a possible synthesis pathway of cilostazol. It can be seen that sodium azide is used for forming the tetrazole ring [4]. Sodium azide is toxic and genotoxic. Its lowest lethal dose is 10 mg/kg. After poisoning, death can occur within an hour by hypotension [5].

According to the European Medicine Agency (EMA) and International Council for Harmonization (ICH), the maximum daily intake of potential genotoxic impurities for more than 12 months of exposure is 1.5 µg. The limit concentration of genotoxic impurity depends on the daily dose of API [6] that varies depending on whether it is used alone or in combination with other anti-platelet agents [1]. If it is taken alone the daily dose is 200 mg/day, if it is taken with CYP2C19 inhibitors it is 100 mg/day [7]. Considering the daily dose mentioned before, this means that the permitted sodium azide concentration is only 7.5 ppm.

Figure 1. Possible synthesis path of cilostazol API.

Due to its low limit concentration, determination of sodium azide is a problem in APIs and pharmaceutical products. Vinkovic and Drevenkar developed an ion chromatographic method for azide determination in protein samples [8]. The USP refers to a method for determining azide impurity in ibersartan API, however, the sample preparation is not applicable for other APIs in general. In this method [9], azide anions are separated by ion chromatography with 0.1 N sodium hydroxide eluent and conductivity detector. The specified column (L31) is a strong anion exchange column with quaternary amine groups [10]. This method was applied for the determination of sodium azide in a range of “sartan” drugs. The sodium azide was quantified at 15 ppm level while the retention time of azide anion was about 42 min [11]. A reversed phase liquid chromatography method was developed for sartan APIs by Gricar and Andresek [12]. In this RP-HPLC method, UV detection was used for determination of azide at 10 ppm limit level. During the sample preparation, the APIs were precipitated and removed by filtration. In case of cilostazol the limit is lower, and the sample preparation should be carried out in different solution. In order to control this impurity at 7.5 ppm level, a large amount of the sample must be dissolved. This high cilostazol API concentration can decrease the efficiency of the analytical method by overloading the chromatographic system. Therefore, during the sample preparation, the amount of the API needs to be decreased while the quantity of sodium azide should remain the same.

The aim of this work was to develop and validate an analytical procedure for the determination of sodium azide content in cilostazol API. In our method, 500 mg cilostazol is dissolved in 5 mL of methylene chloride. Since sodium azide is hydrophilic and cilostazol is insoluble in water, aqueous phase liquid–liquid extraction (LLE) was used for the sample preparation followed by the ion chromatographic determination of the extracted azide anions. The validation was carried out in accordance with ICH Q2(R1) guideline recommendations [13].

2. Materials and Methods

Analytical grade dimethyl sulfoxide, methylene chloride (Fisher Scientific, Loughborough, UK) and sodium azide (Sigma-Aldrich, Darmstadt, Germany) was used for sample preparations. Cilostazol originates from the synthesis of Egis Pharmaceuticals Plc. Water was prepared freshly using ELGA Purelab system (ELGA, Lane End, UK). Mettler Toledo analytical balances were used for weighing (Greifensee, Switzerland) and Eppendorf automatic pipettes were used for liquid handling (Hamburg, Germany).

Dionex ICS 5000 HPIC system equipped with eluent generator (EGC) and suppressed conductivity detector (CD) was used for IC measurements (Thermo Scientific, Waltham, MA, USA). The anion exchange column was Dionex IonPac AS11HC (2 × 250 mm) with a guard column AG11HC (2 × 50 mm) (Thermo Scientific, Waltham, MA, USA). The chromatograms were processed with Chromeleon 7. (Thermo Scientific, Waltham, MA, USA).

3. Results

3.1. Sample Preparation with Liquid-Liquid Extraction

Because of the low concentration limit of sodium azide, large amount of cilostazol API is needed for the determination. The high concentration of API may have a negative impact on the ion chromatographic determination of azide ion. Cilostazol can precipitate in the eluent and deteriorate the column performance or overload it. A suitable sample preparation technique should be developed to avoid overloading problems. Due to the significantly different solubilities of cilostazol and sodium azide in the immiscible solutions of methylene chloride and dimethyl-sulfoxide/water, liquid-liquid extraction can be used efficiently for sample preparation. The methylene chloride solution is the lower phase and the aqueous solution is the upper phase. Then, 500 mg cilostazol is dissolved in 5 mL of methylene chloride (100 mg/mL). The sodium azide is extracted by 5 mL of dimethyl-sulfoxide/water. The extraction efficiency was tested on three temperatures (20, 25, and 30 °C). It was found that the extraction efficiency did not depend on the temperature. Even if the temperature affected slightly the solubility of cilostazol in the aqueous phase, it did not affect the extraction of the azide anion.

3.2. Ion Chromatographic Analysis of Sodium Azide

3.2.1. Effect of Flow Rate

The examined flow rates were over the optimum velocity. However, the results met the system suitability requirements, and the theoretical plates did not change significantly at different flow rates. Accordingly, so either flow rate could be applicable.

3.2.2. Effect of Eluent Concentration

The retention factor azide anion was determined at different eluent concentrations (5, 10, 15, 20, 25, and 30 mM). Figure 2 shows that as retention factors of azide anions decreased by the increasing concentration of the eluent. In ion exchange chromatography, the plot of logarithm (with base 10) of retention factor *versus* logarithm of eluent concentration should be linear for isocratic separations. The slope of equation (-0.989) that is fitted for the measured data points verifies that the retention behavior of azide ion is in line with equilibrium theory.

Figure 2. Logarithm (with base 10) of retention factor of azide anions as a function of logarithm of eluent concentration.

3.2.3. Effect of Column Temperature

The effect of column temperature on the separation of azide anions were studied in the range of 25 °C to 40 °C in six points. The results showed that the azide ion retention time did not depend significantly on the column temperature. The relative standard deviation of the six retention times (measured on six different column temperature) was only 0.18%.

3.3. Validation of the Chromatographic Method

A developed and optimized method can only be used for quantitative measurements of raw materials, intermediates or APIs if the applicability of the method was proved earlier so the method is validated. The validation of this method was performed according to ICH Q2(R1) guideline [13] for limit tests.

3.3.1. Solvent Preparations

The following solutions were used during chromatographic method validation:

- Blank: mixture of 5 mL purified water and 5 mL methylene chloride. It was homogenized by shaking for at least one minute;
- Sodium azide solution: 75 µg/mL of sodium azide in dimethyl sulfoxide;
- Sodium azide reference solution: 5 mL of methylene chloride and 50 µL of the sodium azide solution was added into a HS vial. After the dissolution, 5 mL of purified water was added. It was homogenized by shaking for at least one minute. For the measurement the upper phase was used;
- Test solution: 100 mg/mL of cilostazol in methylene chloride. After the dissolution 5 mL purified water was added. It was homogenized by shaking for at least one minute. For the measurement the upper phase was used;
- Spiked test solution: 500 mg of cilostazol and 50 µL of the sodium azide solution was measured into 5.0 mL methylene chloride. After the dissolution 5 mL purified water was added. It was homogenized by shaking for at least one minute. For the measurement the upper phase was used;
- Limit of detection: 5 mL of methylene chloride and 50 µL of 22.5 µg/mL of sodium azide solution was added into a HS vial. After the dissolution 5 mL purified water was measured. It was homogenized by shaking for at least one minute. For the measurement the upper phase was used.

3.3.2. Ion Chromatographic Method for Azide Determination

As a result of the preliminary experiments the final anion chromatographic method can be seen in Table 1.

Table 1. HPIC method parameters.

Run time	40 min
Eluent	KOH solution
Eluent flow rate	0.50 mL/min
Gradient program	15 mM (5 min) $\xrightarrow{3\text{min}}$ 80 mM (12 min) $\xrightarrow{3\text{min}}$ 15 mM (17 min)
Temperatures	
Autosampler temperature	15 °C
Column temperature	40 °C
CD detector cell temperature	35 °C
Compartment temperature	30 °C
Suppressor	19 mA (7 min) $\rightarrow$ 99 mA (18 min) $\rightarrow$ 19 mA (15 min)
Injection volume	5.0 µL

3.4. Validation Measurements

The following measurements were carried out during limit test validation:

- Specificity. The specificity test has to be made for the limit validation. It can verify that the method is specific and selective for sodium azide;
- Limit of quantification (LQ) and limit of detection (LD). Limit of detection (LD) and limit of quantification (LQ) were specified as the minimum concentration, at which the signal of the investigated component was at least three times (LD) and ten times (LQ) greater than the noise level;
- System precision. System precision was demonstrated by calculating the repeatability of six replicate injections of the reference solution at limit level;
- Accuracy and stability. In the study of accuracy, the sample was spiked with sodium azide at limit level (7.5 ppm). The concentrations were determined, and the recoveries of the spiked quantities were calculated in each case.

The stability was determined by analyzing the prepared solutions over a period of 24 h in closed plastic vials in the sampler holder.

Results of validation are presented in Table 2. Figure 3. shows that no interference from blank and peak due to any impurity was observed at the retention time of sodium azide peaks. The sodium azide reference solution and sample solution stability were measured and the result was, that these were stable for 24 h in the sampler holder. The method usefulness was also proved by measuring four consecutive production batches.

Table 2. Parameters and results of the validation of method developed for the analysis of sodium azide.

Parameters	Results	Requirement
Specificity (ppm)	7.5	7.5
Retention time (t_R , min)	4.41	–
Retention factor (k ; $t_0 = 1.5$ min)	2.94	1–10
Plate number	5097	2000
Symmetry factor (As)	1.1	1.5
Limit of Detection ($S/N = 3$)	0.52	2.25
Limit of Quantification ($S/N = 10$)	1.73	7.5
System precision (at 7.5 ppm)		
Retention time (RSD%)	0.14	5
Peak area (RSD%)	16.5	20
Recovery (% , at 7.5 ppm)	102.4	75–125

Figure 3. Representative chromatograms (1. Blank solution, 2. Limit solution, 3. Spiked sample solution, 4. Sample solution).

4. Conclusions

A fast and effective high performance ion chromatographic (HPIC) method was developed for the determination of sodium azide content of cilostazol API. For the appropriate detection, a liquid–liquid extraction (LLE) step was necessary. The proposed new HPIC method developed for quantitative determination of sodium azide in cilostazol drug substance is accurate, precise, robust and selective. The solutions, which were made with LLE sample preparation, are stable at least 24 h. The method produced satisfactory validation data for the tested parameters for the appropriate ICH guidelines. The developed method is simple, cost-effective, and provides the possibility to reduce the limit concentration by up to three quarters from the current 7.5 ppm, if necessary.

Author Contributions: Conceptualization, R.K. and B.P.; methodology, B.P.; software, K.H.; validation, B.P. and Z.G.; writing—original draft preparation, B.P. and R.K.; writing—review and editing, K.H.; visualization, B.P. and K.H.; supervision, R.K. and K.H.; project administration, K.H.; funding acquisition, K.H. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the Cooperative Doctoral Programme granted by the Ministry for Innovation and Technology from the source of the National Research, Development and Innovation Fund. Financial support of the Hungarian National Research, Development and Innovation Fund (NKFIH FK128350) is also greatly acknowledged.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Abbreviations

The following abbreviations are used in this manuscript:

MDPI	Multidisciplinary Digital Publishing Institute
DOAJ	Directory of open access journals
API	Active pharmaceutical ingredient
CD	Conductivity detector
EMA	European medicine agency
EGC	Eluent generator cartridge
HPIC	High-performance ion chromatography
HPLC	High-performance liquid chromatography
IC	Ion chromatography
ICH	International Council for Harmonization
LD	Limit of detection
LQ	Limit of quantification
LLE	Liquid–liquid extraction
QbD	Quality by design
RP-HPLC	Reversed-phase high-performance liquid chromatography
UV	Ultra-violet

References

1. Cilostazol Monograph for Professionals. American Society of Health-System Pharmacists. Available online: <https://www.drugs.com/monograph/cilostazol.html> (accessed on 5 February 2021).
2. Gysi, D.; Do Valle, I.; Zitnik, M.; Ameli, A.; Gan, X.; Varol, O.; Ghiassian, S.; Patten, J.; Davey, R.; Loscalzo, J.; et al. Network medicine framework for identifying drug-repurposing opportunities for COVID-19. *Proc. Natl. Acad. Sci. USA* **2021**, *118*, e2025581118. [CrossRef] [PubMed]
3. Hiatt, W.; Money, S.; Brass, E. Long-term safety of cilostazol in patients with peripheral artery disease: The CASTLE study (Cilostazol: A Study in Long-term Effects). *J. Vasc. Surg.* **2008**, *47*, 330–336. [CrossRef] [PubMed]

4. Kleemann, A. Cardiovascular Drugs. In *Ullmann's Encyclopedia of Industrial Chemistry*; American Cancer Society: Atlanta, GA, USA, 2008. [CrossRef]
5. Chang, S.; Lamm, S. Human health effects of sodium azide exposure: A literature review and analysis. *Int. J. Toxicol.* **2003**, *22*, 175–186. [CrossRef] [PubMed]
6. ICH Guidance for Industry M7(R1), Assessment and Control of DNA Reactive (Mutagenic) Impurities in Pharmaceuticals to Limit Potential Carcinogenic Risk. 2017. Available online: https://database.ich.org/sites/default/files/M7_R1_Guideline.pdf (accessed on 27 July 2021).
7. European Medicines Agency, Cilostazol-Containing Medicines, Cilostazol-Containing Medicines—Article-31 referral—Annex III (Cilostazol), Published: 11 September 2013. Available online: https://www.ema.europa.eu/en/documents/referral/cilostazol-containing-medicines-article-31-referral-annex-iii-cilostazol_en.pdf (accessed on 27 July 2021).
8. Vinković, K.; Drevenkar, V. Ion chromatography of azide in pharmaceutical protein samples with high chloride concentration using suppressed conductivity detection. *J. Chromatogr. B* **2008**, *864*, 102–108. [CrossRef] [PubMed]
9. United States Pharmacopeia. *The National Formulary. Irbesartan. USP 31-NF 26*; United States Pharmacopeia: Rockville, MD, USA, 2008; p. 2446.
10. USP Monographs for Dionex Columns (2008 USP Pharmacopeia). Available online: <http://tools.thermofisher.com/content/sfs/manuals/Man-025203-USP-Monographs-Man025203-EN.pdf> (accessed on 27 July 2021).
11. Kushwah, D.K.; Kohle, P.Y.; Joshi, R.D.; Rajyaguru, B.; Pandey, R.; Vishwakarma, B. Validated Method for the Quantification of Sodium Azide in a Range of 'Sartan' Drugs by Ion Chromatography. *Res. J. Pharm. Tech.* **2010**, *3*, 82–86.
12. Gričar, M.; Andrenšek, S. Determination of azide impurity in sartans using reversed-phase HPLC with UV detection. *J. Pharm. Biomed. Anal.* **2016**, *125*, 27–32. [CrossRef]
13. ICH Guidance for Industry Q2(R1), Validation of Analytical Procedures: Text and Methodology. 2005. Available online: [https://database.ich.org/sites/default/files/Q2\(R1\)Guideline.pdf](https://database.ich.org/sites/default/files/Q2(R1)Guideline.pdf) (accessed on 27 July 2021).