

The Optimization of the Method of Analysis of Ciprofloxacin, Aciclovir and Cyanocobalamin to Ensure Quality Control in the Developed Domestic Ophthalmic Dosage Forms

Elena T. Zhilyakova, Marina Y. Novikova, Oleg O. Novikov,
Dmitriy I. Pisarev and Alexandra V. Baskakova
Belgorod State University, Pobeda Street 85, 308015 Belgorod, Russia

Abstract: An original method for analyzing substances, ciprofloxacin, acyclovir and cyanocobalamin with which it will be possible to carry out a study of ophthalmic dosage forms containing these components. This is used converts-phase chromatography option. As eluting system to study components chosen mixture of 1% aqueous formic acid-ethyl alcohol in a ratio of 10:90. Since, all components are well or moderately soluble in alcohol, using a mobile phase of 90% of the solvent yielded the optimum retention time of the compounds. Another important advantage of this system is that carried out the replacement of expensive and toxic acetonitrile is traditionally used in the world practice in the method converts phase HPLC on non-toxic and available ethyl alcohol. The quality of the results of chromatography is not treason. To register the results used diode-array detector, registering the electronic absorption spectra which is an objective in the case of the analysis of these compounds because they all have characteristic absorption bands in the UV and visible regions of the spectrum. Analysis time was no >3 min which is a characteristic of the chromatographic process to be very rapid.

Key words: Infections ophthalmic diseases, ciprofloxacin, acyclovir, cyanocobalamin, high performance liquid chromatography

INTRODUCTION

Viral eye diseases hold a specific place among other ophthalmic pathologies because of polymorbidity and polyetiologic pathogeny of visual organ. According to statistical data of Ministry of Health Care and Social Development of the Russian Federation, viral conjunctivitis accounts for about one third of total ophthalmic pathologies it comes up to circa 60% of causes for visiting an ophthalmologist (Commissarov *et al.*, 2013).

One of the manifestations of eye infection is polymorbidity that comes from primary viral infection, accompanied by the formation of follicles. Secondary bacterial or fungal colonization of mucous membranes takes place at the same time this requires the inclusion of repair and antioxidant agents in the eye drops for successful therapy (Kuzmicheva *et al.*, 2013). As an optimal reparative agent we selected cyanocobalamin which has pronounced activity and stimulates the synthesis of purine nucleoside. Another manifestation of secondary ocular infections is a fungal infection of the mucous membrane in the damaged eye. Bacterial infection

of the mucous membrane of the eye is also possible that necessitates the inclusion of an antimicrobial agent. Ciprofloxacin has been carefully selected according to the microbiological testing.

The development of modern complex formulation for treatment of infectious ophthalmic diseases can't be achieved without application of modern analysis method. The High Performance Liquid Chromatography (HPLC) is one of the most efficient and versatile methods of analysis to date.

Currently this chromatographic method has established itself as one of the most effective for the separation and analysis of pharmaceutical facilities for several reasons. The method is multi-source information that is able to provide the results about the authenticity, purity and assay of the active substance. In addition, it is very flexible which characterizes the ability to determine the heat-sensitive and low-volatile substances. Therefore, HPLC perfectly fulfills all the requirements of the analytical methods for the selectivity and sensitivity.

Considering methods for analyzing of ciprofloxacin in the recent literature, it has been found that the HPLC method is used all of the world pharmacopeias. The results are shown in Table 1.

Table 1: Comparative characteristics of methods of analysis by HPLC of ciprofloxacin in various pharmacopeias of the world

Conditions of chromatography						
Pharmacopeia, country	Mobile phase	Column	The flow rate, sample volume	Detector, wave length	Amount of impurities	Retention time
State pharmacopeia XII, 2007 (data are absent)						
BPh (Belorussian Pharmacopeia in 2009)	Acetonitrile P: a solution of 2.45 g L ⁻¹ of phosphoric acid P to pH 3.0 with triethylamine P, (13:87 o6/o6)	The 0.25 m with an inner diameter of 4.6 mm, filled with octadecyl silica gel with particle size of 5 microns	1.5 mL min ⁻¹ , 50 mL	UV 278 nm	Total amount of additional impurities no >0.5%	Double retention time of ciprofloxacin
BP (British Pharmacopeia, 2013)	A mixture of 13 volumes of acetonitrile and 87 volumes of a 2.45 g L ⁻¹ solution of phosphoric acid to pH 3.0 with triethylamine	Column 25 sm x4.6 mm octadecyl silica gel chromatography to 5 microns, column temperature of 40°C	1.5 mL min ⁻¹ , 50 mL	UV 278 nm	Amount of impurities no >0.5%	Double retention time of ciprofloxacin
USP 36 (USA Pharmacopeia in 2014)	Mobile phase acetonitrile and 0.025 M phosphoric acid, bring with triethylamine to pH 3.0±0.1 (13:87)	4.6mmx25 sm particles L1, temperature of column 30°C	1.5 mL min ⁻¹ , 10 mL	UV 278 nm	No >1.5%	The retention time of ciprofloxacin 6.4-10.8
EPh (European Pharmacopeia in 2014)	13 volumes of acetonitrile and 87 volumes of a 2.45 g L ⁻¹ phosphoric acid solution, pH 3.0 with triethylamine	Column length 25 cm x4.6 mm with octadecyl silica gel chromatography 5 mi cron, temperature 40°C	1.5 mL min ⁻¹ , 50 mL	UV 278 nm	No >0.5%	Double tretention time of ciprofloxacin

Table 2: Comparative characteristics of methods of analysis by HPLC of cyanocobalamin in various pharmacopeias of the world

Conditions of chromatography						
Pharmacopeia, country	Mobile phase	Column	The flow rate, sample volume	Detector, wave length	Amount of impurities	Retention time
National pharmacopeia of Russia XII, 2007 (data are absent)						
BPh (Belorussian Pharmacopeia in 2009)	26.5 volume mixture of methane P and 73.5 g of a solution of 10 volumes/disodium hydrogenphosphate P, adjusted to pH 3.5 with phosphoric acid, P (solution unfit for 2 days)	0.25 m and an inner diameter of 4 mm, packed with silica gel for chromatography with a particle size of P 5 microns	0.8 mL min ⁻¹ , 30 mkl	UV, 361 nm.	Amount of impurities no >3%	3: Retention time of cyanocobalamin
BP (British Pharmacopeia in 2013)	Mobile phase from 26.5 volume methane and 73.5 volumes of 10 g L ⁻¹ solution of potassium dihydrogen phosphate to pH 3.5 with phosphoric acid (shelf life of 2 days)	Column 25 cm x4 mm silica gel chromatography oktadetsionym 5 microns	0.8 mL min ⁻¹ , 20 mkl	UV, 361 nm	Amount of impurities no more 3%	3-Retention time of cyanocobalamin
EPh (European Pharmacopeia in 2014)	26 volumes of methane and 75.5 volume sodium hydrogenphosphate to pH 3.5 with phosphoric acid (solution 2 days pass)	Column length 25 cm, width of 4 mm, stationary phase octadecyl silica gel chromatography to 5 microns	0.8 mL min ⁻¹ , 20 mkl	361 nm, UV	Amount of impurities no more 3%	3: Retention time of cyanocobalamin

As seen from Table 1, the requirements for analysis of ciprofloxacin are similar in major pharmacopeias. The information about ciprofloxacin analysis by HPLC is omitted in State Pharmacopoeia XII.

The number of theoretical plates which characterizes the quality of the column used puts as a measure of system suitability only in USP 36. The column temperature to 30°C Celsius changed somewhat in the USP 36 in comparison with other pharmacopoeias and also outputs the peak time interval of ciprofloxacin accurately regulated.

For the analysis of cyanocobalamin the HPLC Method is used in the world's major pharmacopeias. Comparative analysis is presented in Table 2.

As can be seen from Table 2, the requirements analysis of cyanocobalamin are identical in all pharmacopoeias. The European Pharmacopoeia 2010 regulates by several different content volume of methanol and sodium hydrogen phosphate in the composition of the mobile phase. The British Pharmacopoeia 2012, regulates the inclusion of mobile phase of potassium hydrogen phosphate. However, the pH of the solutions in the three methods considered remains unchanged. Also, none of the discussed pharmacopoeias not describe for the number of theoretical plates of the peak of cyanocobalamin.

During the study of scientific and patent literature it was found that the most useful method of analysis

Table 3: Comparative characteristics of methods of analysis by HPLC of aciclovir in various pharmacopoeia of the world

Pharmacopoeia, country	Conditions of chromatography			Detector, wave length	Mobile phase	Retention time
	Mobile phase	Column	Mobile phase			
National Pharmacopoeia of Russia XII in 2007	6.0 sodium dihydrogen phosphate, sodium disulfonate 1.0-pH 3+/-1 phosphoric acid, 40 mL of acetonitrile and 1000 mL of water	The length of 0.10 m, inner diameter 4.6 mm silica 3 microns Chromatography	2 mL min ⁻¹ injection 20 mL	UV, 254 nm	Not >0.7% of guanine Not >0.5% Any other impurity	7-retention time of aciclovir
Ucrainian Pharmacopoeia in 2007	No differens from the Russian Pharmacopoeia XII					
BPh (Belorussian Pharmacopoeia in 2009)	No differens from the Russian Pharmacopoeia XII					
BP (British Pharmacopoeia in 2013)	No differens from the Russian Pharmacopoeia XII					
USP 36 (USA Pharmacopoeia in 2014)	The solution was filtered and acetic acid was degassed (1: 1000)	Length 0.25 m the inner diameter of 4.6 mm sorbent type L1 (capsule internal diameter 4.2 mm)	3 mL min ⁻¹ injection 20 mL (in the injection solution of 1 mL min ⁻¹) for the capsules of 1.5 mL min ⁻¹	Spectrophotometry, 254 nm	Guanine not >0.7%	Retention time is 56 min
JP 16 (Japan Pharmacopoeia in 2006)	Mobile phase A: Acetonitrile R, phosphate buffer solution, pH 2.5	Column length of 0.15 m, gel chromatography inner diameter 4.6 mm Sorbent is octadecyl silica	3 mL min ⁻¹ Sample volume 5 mL	Spectrophotometry, 254 nm	Guanine not >0.7%	Retention time is 7 min
EPh (European Pharmacopoeia in 2014)	Solvent mixture: DMSO R, R water (20:80 V/V) Fosfatny buffer pH 2.5. 3.48 g of dipotassium hydrogen phosphate R in 1000 mL of water R to pH 2.5 with phosphoric acid to pH 3.1	Column length of 0.25 m, inner diameter 4.6 mm sorbent is octadecyl silica gel chromatography, 5 m	1 mL min ⁻¹ sample volume 10 µL	UV, 254 nm	Guanine not >0.7%	Retention time is 7 min

of aciclovir is High Performance Liquid Chromatography (HPLC) in various dosage forms in universal practice.

The requirements for the analysis of aciclovir are somewhat different in regulatory documents of different countries. Thus, the State Pharmacopoeia XII, 207 provides a method of reverse phase HPLC for the substance of aciclovir. Methods of analysis haven't any differences which shown in Ukrainian Pharmacopoeia the Belarussian Pharmacopoeia the British Pharmacopoeia (British Pharmacopoeia Commission, 2012). In the BPh and BP some possible impurities are described in the substance of aciclovir as well as in Japanese and the American (USP, 2012) pharmacopoeias.

Table 3 shows the comparative characteristics of HPLC conditions for the quantitative determination of acyclovir.

As can be seen from Table 3, HPLC is the most useful method for analyses of aciclovir in the different dosage forms in these countries requirements.

The European Pharmacopoeia 2014 involves the use of column of 25 cm × 0.46 against the claims in the State Pharmacopoeia XII 10 × 0.46 cm. It is also characterized by

the flow rate per minute: 1-2 mL, respectively and different sample volume, constituting 10 Mcl Eph solution and 20 mcL of the State Pharmacopoeia XII. The use of sodium phosphate in acetonitrile against potassium dihydrogen phosphate in dimethyl sulfoxide and acetonitrile was presented as a component of the mobile phase in the State Pharmacopoeia XII. Thus, we can see that no differences between requirements for HPLC in State Pharmacopoeia XII, 2007 and the BP in 2009.

Methods of the State Pharmacopoeia XII and 29 USP are differ among themselves in the length of mentioned chromatographic column. The conditions are the same for USP 29 and Eph. The velocity of flow according to the requirements of USP 29 is 3 mL/min in contrast to other methods. When the flow rate for the various dosage forms is variable because of the chemical and technological characteristics of the preparation.

For small velocity of flow the solvent for injection of aciclovir is characterized by the need to particularly high precision separation of the peaks in the chromatogram, it is possible to extend the time of chromatography (Zhilyakova *et al.*, 2014).

Thus, in analyzing the various pharmacopoeias of the chromatographic conditions of studied objects, the conclusions have been obtained:

- A mixture of phosphates and acetonitrile is used as mobile phase. However, it is known that the phosphates in the interaction with multiply charged cations that may be part of a mobile system form insoluble salts which can deposit on parts chromatograph reducing its service life
- The acetonitrile is sufficiently toxic components in the mobile phase
- Each pharmacopoeia recommends own conditions for the analysis that does not allow unifying the conditions for research of the test components

Therefore, we attempted to optimize the method of analysis of the test components to ensure quality control as a part of ophthalmic dosage forms.

As components of the mobile phase, we used formic acid as a component which provides no precipitation with multiply, charged metal cations. We have replaced the toxic acetonitrile to ethyl alcohol.

MATERIALS AND METHODS

The chromatographic studies were carried out on chromatographic instruments «Agilent Technologies 1200 Infinity» with autosampler “Agilent 1200 Series” and vacuum degasifier, gradient pump and thermostat with the same series. Electronic absorption spectra were recorded using a spectrophotometric diode-array detector Agilent series 1200 (a wavelength range was of 190-950 nm in a cuvette with a path length of 10 mm; volume 13 μ L), the scanning step was 2 nm.

The software «Agilent Chem Station” was used for the registration and processing of spectral data and chromatograms.

The steel chromatographic column Ascentis express C18 2.7 μ m \times 100 mm \times 4.6 mm was used in the tests. The ultrapure water (liquid chromatography), ethyl alcohol (GOST R 51652), formic acid as the solvents were used to prepare mobile phase.

The column efficiency was determined by calculating the theoretical plate number N. High column efficiency characterizes by the number of theoretical plates. The peak broadening is smaller relatively narrow band as it moves through the column and the peak narrows at the output. The value is not <5000 theoretical plates are used as a criterion for the optimal column efficiency.

The main criterion for assessing the adequacy of the separation of adjacent peaks served as the separation factor R_s which shall not be <1.5 according to the European Pharmacopoeia. Wherein the peaks are separated by a baseline.

The shape of the chromatographic peak which characterizes overload chromatographic column was determined by calculating the ratio of the peak asymmetry (T_p). The optimal size of the asymmetry coefficient T_f accepted figure is <2.

The control system suitability: The chromatographic system may be considered suitable if the following conditions are achieved:

- The effectiveness of the chromatographic system designed for peaks should be not <5000 theoretical plates
- The peak asymmetry factor should be <2
- The relative standard deviation of the peak areas in the chromatograms of the test solution should be:
 - From two parallel chromatograms no >0.51%
 - For the three chromatograms No. >1.34%
 - For the four chromatograms No. >1.92%
 - For the five chromatograms No. >2.37%

Preparation of test solution: The 0.1 g of the test substance was placed in a volumetric flask with volume of 100.0 mL. A suitable solvent was added according to the physics and chemicals properties. Cyanocobalamin was dissolved in purified water. Ciprofloxacin and aciclovir were dissolved in 0.1 N aqueous solution of hydrochloric acid in a volume of 20.0 mL, further stirred up to full dissolution and was adjusted with the same solvent up to the mark.

In operation, the optimal conditions were determined for the chromatographic separation of analytes:

- Mobile phase: 1.0% aqueous formic acid-ethyl alcohol (10: 90)
- Rate of mobile phase: 0.5 mL/min
- Column temperature: 35°C
- The volume of injected sample: 1 μ L
- Detection: diode array

RESULTS AND DISCUSSION

Figure 1 shows the chromatogram of ciprofloxacin substance. Ciprofloxacin provides a chromatographic peak with a retention time of 1.114 min.

The detection of ciprofloxacin was performed at a wavelength of 278 nm, since this area is its absorption maximum (Fig. 2 and 3).

The eligibility criteria have been calculated to confirm the suitability of the system. The results are presented in Table 4.

The detection cyanocobalamin was carried out at a wavelength of 361 nm, due to the presence in its structure of the corrin system with the most pronounced absorption maximum (Fig. 4). The criteria for system suitability are presented in Table 5.

Thus, the eligibility criteria indicate that the chromatographic system is suitable for the determination of cyanocobalamin.

Figure 5 shows the chromatogram of aciclovir substance. Aciclovir have a chromatographic peak with a retention time of 1,605 min.

Detection of aciclovir was carried out at a wavelength of 252 nm which is typical for the structure of guanine (Fig. 6). The indicators of system suitability for determining of aciclovir are presented in Table 6. The

Table 4: The indicators system suitability to determine the substance of ciprofloxacin

t_R	S	N	T_f	W_b
1.114	308.1	8950.5	1.038	0.0471

Table 5: The indicators of system suitability to determine the substance of cyanocobalamin

t_R	S	N	T_f	W_b
2.602	640.4	19155.7	0.664	0.0752

t_R : the absolute retention time, S: peak area, N: the number of theoretical plate, T_f : coefficient of asymmetry, W_b : peak width at the baseline

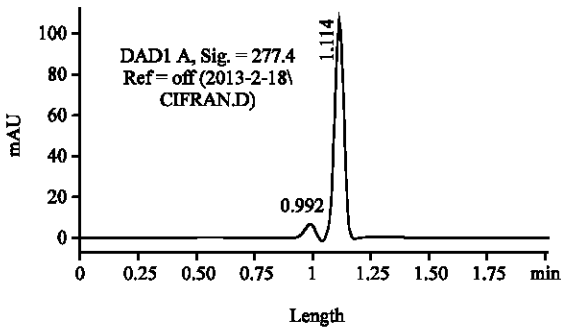


Fig. 1: The chromatogram of substance of ciprofloxacin

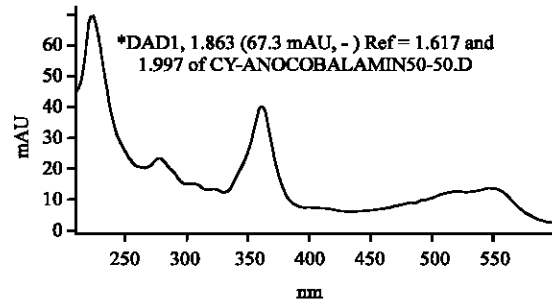


Fig. 4: The UV spectrum of substance of cyanocobalamin

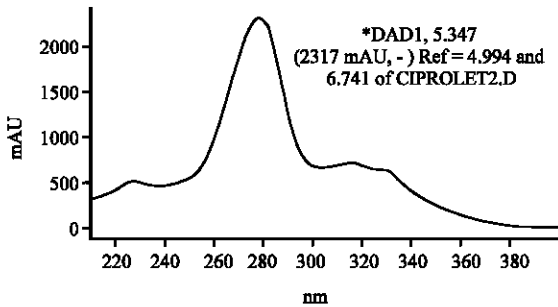


Fig. 2: The UV spectrum of substance of ciprofloxacin

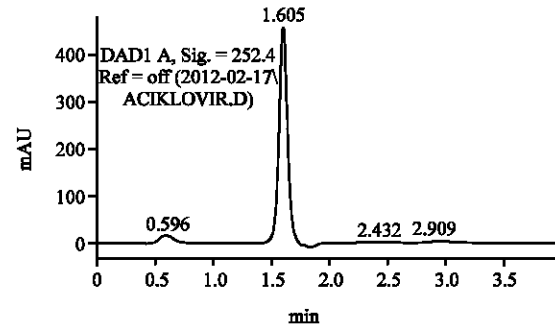


Fig. 5: The chromatogram of substance of aciclovir

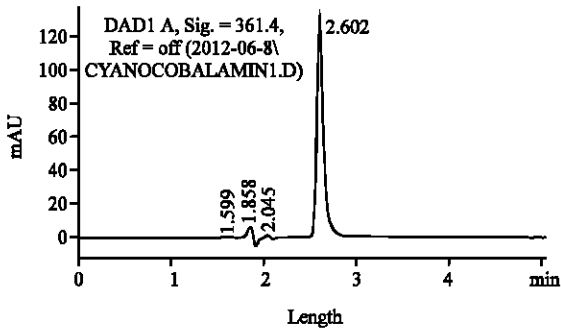


Fig. 3: The chromatogram of substance of cyanocobalamin

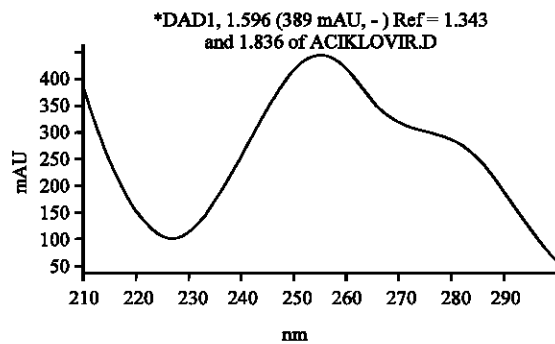


Fig. 6: The UV spectrum of substance of aciclovir

Table 6: The indicators of system suitability to determine the substance of aciclovir

t_R	S	N	T_f	W_b
1.605	2452.6	6488.6	0.915	0.0797

t_R : the absolute retention time, S: peak area, N: the number of theoretical plate, T_f : coefficient of asymmetry, W_b : peak width at the baseline

eligibility criteria indicate that chromatographic system used is suitable for the determination of aciclovir. Thus, the original method of analyzing substances has been proposed for ciprofloxacin, aciclovir and cyanocobalamin whereby it will be possible to carry out the study of ophthalmic formulations containing these components. This is used converts-phase chromatography option.

A mixture of 1% aqueous formic acid-ethyl alcohol in a ratio of 10:90 was chosen as eluting system to study components.

Because all components are well or moderately soluble in alcohol, using alcohol 90% in the mobile phase yielded the optimum retention time of the compounds. Another important advantage of this system is that the replacement of expensive and toxic acetonitrile which was traditionally used in the world practice in the method converts, phase HPLC on non-toxic and available ethyl alcohol.

CONCLUSION

The quality of the results of chromatography has changed. Diode-array detector for the registration of the

results was used. The detector registers the electronic absorption spectra it is an objective in the case of the analysis of these compounds. These compounds have characteristic absorption bands in the UV and visible regions of the spectrum. Analysis time was no >3 min which is a characteristic of the chromatographic process to be very rapid.

REFERENCES

- British Pharmacopoeia Commission, 2012. British Pharmacopoeia. Stationery Office Books, UK.
- Commissarov, M.A., E.T. Zhilyakova and A.V. Baskakova, 2013. Development of techniques solubility of acyclovir. *Sci. Statements BSU. Ser. Med. Pharm.*, 25: 38-41.
- Kuzmicheva, O.A., A.V. Zhilyakova and Baskakova, 2013. The comparative characteristic of methods analysis of acyclovir. *Sci. Statements BSU. Ser. Med. Pharm.*, 25: 100-103.
- USP., 2012. The united states pharmacopeia and the national formulary. U.S. Pharmacopeial Convention (USP), Rockville MD., pp: 2041-2042, 3382-3383.
- Zhilyakova, E.T., O.O. Novikov and M.Y. Novikova, 2014. The study of the solubility of aciclovir in the model mixture of solubilizers with beta-cyclodextrin. *Basic Res.*, 9: 2250-2257.