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Quantitive Determination And Validation Of Cobafen (Lyophilizate 10mg For Preparation Of Solution For Injection)

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ABSTRACT

Research objective: to conduct a quantitative determination of sodium diclofenac and vitamin B-12 in new lyophilic drug Cobafen using modern physicochemical methods of analysis.

Materials and methods. During the research we used the samples of commercially available substances of mecobalamin chloride produced by Apex Medichem Ltd. (India), D-mannitol manufactured by Shandong Tianli Pharm. Co. Ltd. (China) and polyvinyl pyrrolidone (PVP) produced by Merck (Germany), as well as chemical reagents by Sigma-Aldrich (USA) and Hi-Media (India). For spectrophotometry analysis we applied a UV-1800 double-beam spectrophotometer (Shimadzu, Japan) and Agilent 8453E single-beam spectrophotometer (Agilent Technologies, Germany). Besides, the study was carried out under conditions of reverse phase HPLC (Agilent 1280 gradient HPLC chromatograph (Agilent Technologies, USA) and LC-20 (Shimadzu, Japan)).

Results. Conducted 5 independent experiments for each formulation and each freezing mode showed that D-mannitol is the additive agent of choice. Besides, lyophilizates produced using D-mannitol corresponded to the indicated quality attribute. When analyzing mecobalamin by HPLC, it was established that the method of choice was PP with methanol: buffer solution (26.5:73.5), the optimal concentration in the analysis of mecobalamin is 2 mg / ml.

Conclusion. It was established that sharp freezing condition is the most preferred due to the saving in production cycle time. The proposed HPLC method for quantitative determination of the active substance in the drug, validated by all validation parameters, is included in the pharmacopoeial monographs of JURABEK LABORATORIES JV LLC.

Key-words: Cobafen, quantitative determination, validation, lyophilizates, freezing.

1. INTRODUCTION

Liophilisation, also known as freeze drying method, is widely used in the pharmaceutical industry for stabilization and improvement of long-term storage stability of drugs, especially in the production of injectable dosage forms. The stability of the drug molecule is a key factor which impacts on the choice of drug form by the manufacturer: injectable drug in liquid or powder form. According to the research results, it was established that the molecules have a different structure and physicochemical properties. Depending on

this property, the scientists distinguish the injection forms into those containing large or small molecules. Non-biological drugs such as Omeprazole, Pantoprazole, Vancomycin, etc., belong to the small molecule category, and Rituximab, Etanercept, etc. are referred to the category of large molecules. Vaccines such as DTP, TABT vaccine (Typhoid), varicella vaccine are classified as large molecules category; as well as probiotics - lactic acid bacteria, bifidobacteria are also referred to large molecules.

In 2016, the US Food and Drug Administration (FDA) approved 104 injectable drugs, of which 43 were in the form of lyophilizate, including the NDA, BLA and ANDA categories. It can be assumed that these approved drugs are replicated or will be replicated around the world. Thus, in the future, lyophilized injections will become promising, and every manufacturing company using basic lyophilization method will achieve meaningful results [5].

At present time, drugs based on cyanocobalamin metabolites - Methylcobalamin and Mecobalamin are widely used in medical practice [1,2]. They present chemically active substances that enter the biochemical processes of the body's vital activity without initial metabolic transformations and have a more pronounced therapeutic effect [3].

However, the high reactivity of these substances allots a task for specialists to determine the optimal dosage form with a minimum of stabilizers and preservatives, which will preserve the therapeutic effect of the vitamin B12 metabolite.

One of these promising manufacturers is JURABEK LABORATORIES JV LLC (Uzbekistan). Today the company produces a lyophilic drug Cobafen, the main components of which are sodium diclofenac and cyanocobalamin. The studies showed that the combination of these medicinal substances with different mechanisms of action can increase the effectiveness of the treatment.

Currently pain is considered a serious factor in the quality of life. In particular, the problem of back pain is extremely urgent, since most of the population throughout the world suffer from it in different periods of their lives. Moreover, 70% of population at least once in life lose the ability to work for this reason. The peak of complaints of back pain falls on the prime of life - middle working age from 30 to 45. In most cases the treatment includes the administration of nonsteroidal anti-inflammatory drugs (NSAIDs) diclofenac sodium, which is a pathogenetic agent for acute and chronic pain associated with tissue damage and inflammation. Vitamin B 12 participates in a number of intracellular processes, providing the health of cells and the production of sufficient energy for the healthy nervous system [5].

RESEARCH OBJECTIVE

The aim of this paper is a quantitative determination of sodium diclofenac and vitamin B-12 in the new lyophilic drug Cobafen using modern physicochemical methods of analysis.

2. MATERIALS AND METHODS

The object of the research is Cobafen, lyophilizate 10 mg for the preparation of solution for injection in vials (with a solvent).

During the investigation we used the samples of commercially available substances of mecobalamin chloride produced by Apex Medichem Ltd. (India), D-mannitol manufactured by Shandong Tianli Pharm. Co. Ltd. (China) and polyvinyl pyrrolidone (PVP) produced by Merck (Germany), as well as chemical reagents by Sigma-Aldrich (USA) and Hi-Media (India). We also applied a UV-1800 double-beam spectrophotometer (Shimadzu, Japan) and Agilent 8453E single-beam spectrophotometer (Agilent Technologies, Germany). Spectra were determined in the wavelength range from 200 to 760 nm. The appropriate solvent was used as a compensation solution.

Besides, the study was carried out under conditions of reverse phase HPLC. We used Agilent 1280 gradient HPLC chromatograph (Agilent Technologies, USA) and LC-20 (Shimadzu, Japan). Column Zorbax (Agilent Technologies, USA) C18 (250 mm x 4.6 mm, 5 μm), Zorbax guard column (Agilent Technologies, USA) (14 mm x 4.6 mm, 5 μm). Column temperature 25 °C. The Table 20 shows the chromatographic conditions.

3. RESULTS AND DISCUSSION

Preparation of samples for freezing: solutions in the amount of 1 ml were poured into 5 ml glass vials (glass class: HC-1) produced by Neo-Tech Plast (Republic of Uzbekistan). Lyophilization of samples and sealing of vials with bromine-butyl stoppers with combined aluminum caps was performed on the automated conveyor equipment Tofflon (China).
Conditions for lyophilization: the lyophilic dryer shelves were kept at -50 °C and pressure in chamber 0.01 mbar during 5 hours. The shelves were heated in the following modes:
- from –50 ° C to –10 ° C at the rate 10 °C/h;
- from -10 ° C to 0 ° C at the rate 3 ° C/h;
- from 0 ° C to +25 ° C at the rate 5 ° C/h.

After the drug reached the temperature +25 ° C (the pressure in the chamber - 0.01 mbar), it was kept at this temperature for 4 hours. In general lyophilization lasted 22 hours.
Physicochemical testing of the finished product and the quantitative determination of the active substance were determined in the chemical laboratory of the Quality Control Department of the pharmaceutical plant JURABEK LABORATORIES JV LLC (Republic of Uzbekistan) in accordance with the requirements of the European Pharmacopoeia [4].
*The appearance o*f the lyophilizate was determined visually.
The solubility of the lyophilizate was determined visually, recording the dissolution time with a stopwatch. A sufficient amount of water for injection was added to the weighed sample of the substance (1 g) or to the contents of the vial, and the mixture was shaken for 3 min.
Determination of weight loss on drying was carried out by drying the preparation in a vacuum drying oven over P_2O_5 at room temperature and residual pressure of 5 mm Hg. to constant weight. The weight loss in all studied samples did not exceed 3.0%.
The clarity of the solution was determined visually comparing the test liquid with the solvent.
The pH of the aqueous solution of the preparation was determined potentiometrically using a Mettler Toledo instrument (USA).
Quantitative determination of mecobalamin was performed by UV spectrophotometry (SP) and HPLC [6,7]
SP method. Despite the fact that to date the leading foreign pharmacopoeias usually apply IR spectroscopy and chromatographic methods to identify substances and preparations, the UV spectrophotometry is also widely used in pharmacopoeial analysis. For substances with good chromophores, this method allows to conduct the analysis on "identification" and "quantitative determination", and in some cases it can also be used in purity analysis by absorption at certain wavelengths. The complex conjugated system in the structure of mecobalamin allows to widely use UV spectrophotometry for the pharmacopoeial analysis.
The determination is performed by the method of absorption spectrophotometry in the UV area.
The optical density of the test solution and work standard of mecobalamin solution was measured on the spectrophotometer, wavelength 351 nm in a cuvette with a layer thickness of 10 mm, using a solvent as the reference solution.
The content of mecobalamin in one vial in percentages of the LC (X) is calculated by the formula:

$$X = \frac{D_1 * m_0 * 5 * 100 * 100 * P * G}{D_0 * m_1 * 100 * 25 * 100 * L} = \frac{D_1 * m_0 * P * G}{D_0 * m_1 * 5 * L}$$

where

D_1 - the optical density of the test solution;

D_0 - the optical density of the work standard of mecobalamin solution;

M_1 - weighed sample of the drug, mg;

M_0 - weighed sample of the mecobalamin work standard, mg;

P - the content of mecobalamin in the mecobalamin work standard, %;

G - the average mass of the vial contents, mg;

L - the label claim of mecobalamin in one vial, mg.

The drug must contain at least 90.0% and not more than 110.0% of the label clay of mecobalamin.

Note. *Solvent*. 10.9 g sodium acetate trihydrate in a 1000 ml volumetric flask is added 8 ml of glacial acetic acid. The mixture is dissolved and diluted to the mark with injection water.

Test solution. About 100 mg of the drug in a 100 ml volumetric flask is diluted with the solvent up to the mark. 25.0 ml of the resulting solution is taken to the 100 ml volumetric flask and diluted with the solvent to the mark.

Standard sample solution. About 50 mg (accurately weighed) of mecobalamin work standard in a 100 ml volumetric flask is dissolved in the same solvent to the mark. 5 ml of the resulting solution is placed into a 100 ml volumetric flask and dissolved with the solvent to the mark.

Quantitative determination of mecobalamin by HPLC. In modern pharmacopoeial analysis, the HPLC method is widely applied for identification, analysis of purity and quantitative determination of drugs. Besides, this method is used in the analysis of substances and medicinal preparations of vitamin B-12. In the pharmacopoeial analysis of mecobalamin, various sample preparation techniques, chromatographic conditions, and different eligibility criteria of the chromatographic system are used.

Quantative determination of mecobalamin using HPLC.

Buffer solution. 10 g of di-sodium hydrogen phosphate monosubstituted (Na_2HP0_4) is dissolved in 800 ml of injection water, the pH is adjusted to 3.5 with orthophosphoric acid, placed in a 1000 ml volumetric flask and diluted with water to the mark with.

Mobile phase. Methanol: buffer solution (26.5:73.5).

Test solution. About 400 mg of the drug is placed in a 100 ml volumetric flask, dissolved in the mobile phase and the volume is brought up to the mark with the same solvent.

Reference solution. About 40 mg of mecobalamin work standard is placed in a 100 ml volumetric flask, dissolved in the mobile phase and the volume of the solution is brought to the mark with the same solvent.

Chromatographic conditions:

Column	25.0 x 0.46 cm, Silica gel for chromatography, 5 μm;
Column temperature	35 ° C;
Flow rate	1.0 ml / min;
Spectrophotometric detector,	361 nm;
Sample volume	20 μL.

The content of mecobalamin in the drug in percentage (X) is calculated by the formula:

$$X = \frac{S_1 * m_0 * 100 * P * G}{S_0 * m_1 * 100 * L} = \frac{D_1 * m_0 * P * G}{D_0 * m_1 * L}$$

where

S_1 - the average value of the peak areas of mecobalamin, obtained from the test solution chromatograms;
S_0 - the average value of the areas peak of mecobalamin, obtained from mecobalamin work standard chromatograms;
M_1 - the sample of the drug, mg;
M_0 - weighted sample of mecobalamin work standard, mg;
P - the content of mecobalamin in mecobalamin work standard, %;
G - the average mass of one vial contents, mg;
L - the LC of mecobalamin in one vial, mg.
The drug must contain min. 90.0% and max. 110.0% of the label clay of mecobalamin.

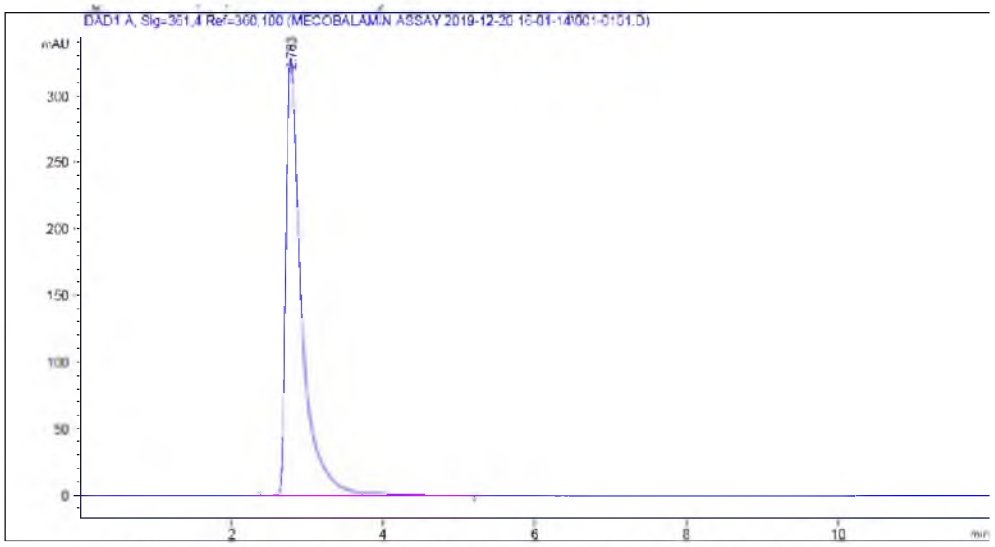


Fig. 1. Chromatogram of mecobalamin standard solution

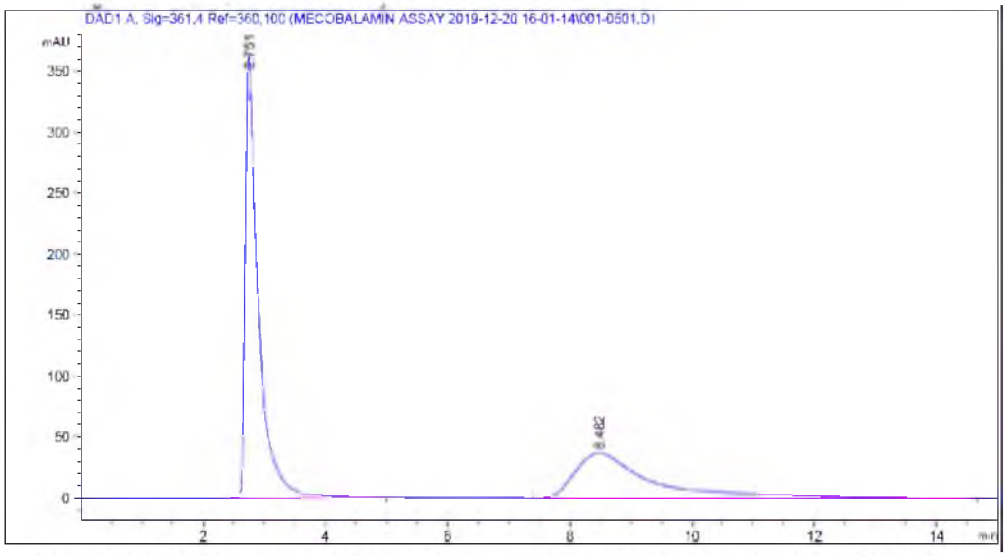


Fig. 2. Chromatogram of mecobalamin test solution in the drug Cobafen

Analytical method validation for the quantitative determination of methylcobalamin in Cobafen, lyophilizate 10 mg for the preparation of a solution for injection.
Analytical method validation is the experimental proof that the method is applicable for its intended purpose.

This paper regulates the characteristics of analytical methods, determined for validation, as well as corresponding criteria for the applicability of validated technologies intended for quality control of drugs and pharmaceutical substances.
Quantitative determination methods, including methods for determining impurities and limit concentration, are subject to validation. Identification methods of drugs are subjected to validation if it is necessary to confirm their specificity.
Validation assesses the analytical method according to the characteristics listed below, selected according to the typical recommendations given in the table:

- specificity;
- detection limit;
- quantitation limit;
- analytical area (range);
- linearity;
- trueness;
- precision;
- robustness.

The specificity of the method is determined by comparing the values of the active ingredient in the analysis of solvent, placebo and drug.

Table 1

Validation parameter	Acceptance criteria
Specificity	Neither the solvents, nor the reagents used in sample preparation, nor the placebo components should not distort the results.
Linearity	Correlation coefficient $\geq$0.99
Within-lab precision	t$\leq$ 2.228 F$\leq$ 5.05
Trueness	Response factor: average 97.5 - 102.5%. Coefficient of Variance $\leq$ 2.0%. Confidence interval should include 100% of values.

Besides, we conducted analysis and measurement of the placebo (reference medicinal product, mixture of all drug ingredients without active ingredient), active ingredient and drug. The Table 2 presents the data obtained:

Table 2

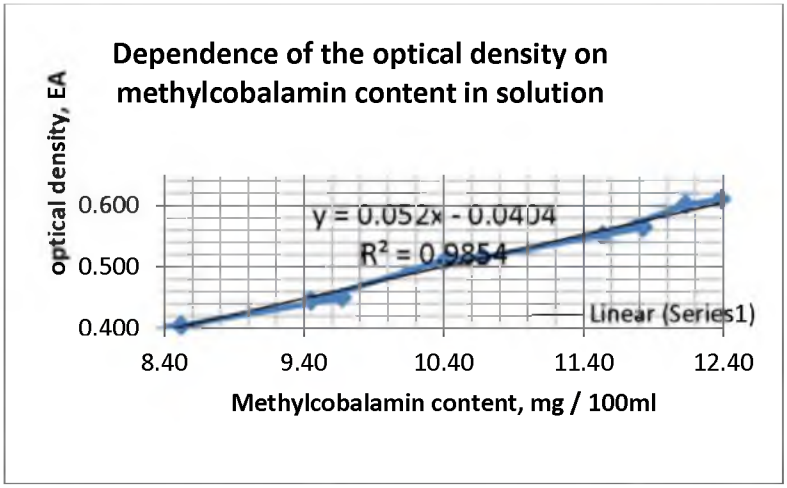
Sample	Optical density
Placebo	0,000
Active ingredient (101 mg / 100ml)	0,512
Medicinal product	0,506

The linearity of the method was determined on min. 5 different dilutions of the test solution in the application range of 80 - 120% concentration of the analyte in the test solution.
We prepared and carried out measurements of drug solutions with the active substance concentration in the interval from 80 to 120%: 2 solutions with the active substance concentration 80%, 2 solutions with the active substance concentration 90%, 2 solutions with the active substance concentration 100%, 2 solutions with the active substance concentration 110% and 2 solutions with the active substance concentration 120%.

Table 3

№	Concentration level,%	Weighted sample, mg	Sod. methylcobalamin in weighted sample, mg	Y (Optical density, EA)
1	80	98,8	8,3200	0,402
2	80	101,2	8,5221	0,404
3	90	103,2	9,6750	0,449
4	90	100,8	9,4500	0,444
5	100	101,1	10,4227	0,512
6	100	103,6	10,6804	0,515
7	110	105,4	11,8306	0,564
8	110	102,8	11,5388	0,551
9	120	100,10	12,1333	0,601
10	120	102,2	12,3879	0,610
average			10,4961	0,5052

Based on the data obtained, the dependence of the optical density on the concentration of methylcobalamin in the solution was determined. The program also draws a trend line and determines the regression equation.



Precision is carried out by testing min. 6 prepared samples at 100% concentration of the active substance in the test solution.

6 solutions of the drug at 100% concentration of the active substance were prepared and taken measurements. The results are shown in the Table 4:

Analyte No. 1. Weighted sample of St 50.2mg, G 100mg, Optical density St 0.504EA

Table 4

No. of sample	Weighted sample, mg	Optical density, EA	Specified content of methylcobalamin%	Xi-Xav	$(Xi-Xav)^2$
1	100,4	0,491	97,4	0,15143348	2,29E-02
2	102,6	0,501	97,3	0,00407434	1,66E-05
3	105,1	0,509	96,5	-0,79343384	6,30E-01

4	100,8	0,491	97,0	-0,23515634	5,53E-02
5	102,9	0,503	97,4	0,10766314	1,16E-02
6	101,6	0,500	98,0	0,76541923	5,86E-01
			$\overline{X} = 97,27$		Σ 1,31E+00

Table 5

Analyte No. 2. Weighed sample of St 51.3mg G 101mg, Optical density St 0.513EA

No. of sample	Weighted sample, mg	Optical density, EA	Specified content of methylcobalamin%	Xi-Xav	$(Xi-Xav)^2$
1	101,3	0,495	98,71	0,283252	8,02E-02
2	98,6	0,482	98,75	0,322891	1,04E-01
3	100,4	0,491	98,79	0,363293	1,32E-01
4	103,1	0,504	98,75	0,323288	1,05E-01
5	102,8	0,496	97,46	-0,960525	9,23E-01
6	100,7	0,489	98,09	-0,332199	1,10E-01
		$\overline{X} = 98,42$		Σ 1,45E+00	

Table 6

Statistical characteristics,%	Results Analyte No. 1	Results Analyte No. 2
Minimum value	96,5	97,46
Maximum value	98,0	98,79
Mean value	97,3	98,42
Standard deviation	0,511	0,539
Coefficient of Variance	0,525	0,548
The lower limit of confidence interval (P = 95%)	96,7	97,86
The upper limit of confidence interval (P = 95%)	97,8	98,99
F(95% $f_1=N_1-1;f_2=N_2-1$) ≤ 5.05	1,11	
t_{kp}(95% and N1+N2-k=10) ≤ 2.228	1,660	
Minimum value	96,5	97,46

Note: No statistically significant differences

The trueness of the method determines the response of the active substance in the placebo. The placebo concentration is 100% of the sample specified in the control method. The active substance is added to the model mixture in accordance with the required concentration level (lower limit - max. 80, 100%; upper limit - min. 120%).

We prepared and carried out measurements of drug solutions with the active substance concentration in the interval from 80 to 120%: 3 solutions with the active ingredient concentration 80%, 3 solutions with the active ingredient concentration 90%, 3 solutions with

the active ingredient concentration 100%, 3 solutions with the active ingredient concentration substances 110% and 3 solutions with the active substance concentration 120%.

Weighted sample St 50.2mg G 100mg, Optical density St 0.504 EA
Table 7

Concentration level, %	Weighed sample of methylcobalamin, mg	Sod. methylcobalamin in the sample	Optical density, EA	Determination of content methylcobalamin, %	Specified content of methylcobalamin,%	Response ,%
80%	98,8	8,3200	0,402	81,1	80,0	101,32
80%	101,2	8,5221	0,404	79,5	80,0	99,41
80%	100,1	8,4295	0,402	80,0	80,0	100,00
100%	101,1	10,4227	0,512	100,9	100,0	100,88
100%	103,6	10,6804	0,515	99,0	100,0	99,03
100%	99,10	10,2165	0,497	99,9	100,0	99,90
120%	100,10	12,1333	0,601	119,6	120,0	99,67
120%	102,2	12,3879	0,610	118,9	120,0	99,08
120%	100,8	12,2182	0,608	120,2	120,0	100,13
Mean value						99,94

Table 8
Statistical processing of the results

Statistical characteristics, %	Results
Mean value	99,94
Standard deviation	0,771
Coefficient of Variance	0,77
Lower limit of the confidence interval (P = 95%)	99,34
Upper limit of the confidence interval (P = 95%)	100,53

4. CONCLUSIONS

1. Obtained data of 5 independent tests for each formulation and each mode of freezing showed that D-mannitol the additive agent of choice.
2. Lyophilizates using D-mannitol as a cryoprotector at concentrations 0.080; 0.100; 0.120 g/vial corresponded to the indicated quality attribute. Lyophilizate reconstitution time containing 0.080 g/vial of additive agent was minimum. In this regard, a composition of hydroxocobalamin chloride with D-mannitol at concentration of 0.080 g/vial is proposed for further research.
3. The freezing condition does not influence on the quality of the lyophilizate. Sharp freezing condition is most preferred due to the saving in production cycle time.
4. UV spectrometric method has been developed to determine the "Quantification" indicator of the drug Cobafen.
5. When analyzing mecobalamin by HPLC, it was established that the method of choice was PP with methanol: buffer solution (26.5:73.5), the optimal concentration in the analysis of mecobalamin is 2 mg / ml.

6. The proposed HPLC method for quantitative determination of the active substance in the drug, validated by all validation parameters, is included in the pharmacopoeial monographs of JURABEK LABORATORIES JV LLC.

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