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PURITY DETERMINATION OF GYNALGIN BACTERICIDAL TABLETS WITH HPLC METHOD

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Abstract: The study was aimed at developing a simple HPLC method for the determination of the content of impurities in Gynalgin, a two-component preparation. A satisfactory separation was performed on 250 × 4.6 mm Symmetry C8 column in a gradient elution system: mobile phase A – acetonitrile/buffer, pH 5.5 in 10:90, v/v proportion, and mobile phase B – acetonitrile/buffer, pH 5.5 in 75:25 v/v proportion. Two wavelengths: 250 nm and 315 nm were used for detection. Validation confirmed that the method was linear in a required concentration range. The values of correlation coefficients for specific drug substances and the related impurities were as high as 0.999. The results of the purity tests proved that the method was sufficiently selective and precise.

Keywords: chlorquinaldol, metronidazole, 2-methyl-5-nitroimidazole, 2-aminophenol, HPLC method

The combined action of multiple drug substances improves therapeutic efficacy, and therefore, the number of multi-component drugs available on the market has been continuously increasing. Gynalgin preparation is available as vaginal tablets and contains two drug substances: chlorquinaldol and metronidazole. Chlorquinaldol is a derivative of 8-hydroxyquinoline exhibiting bactericidal, fungicidal and antiprotozoal activity; metronidazole is a derivative of 5-nitroimidazole with antiprotozoal and bactericidal activity against anaerobes.

The aim of the study was to develop the easiest possible HPLC method to separate and determine the content of impurities in Gynalgin medicinal product.

Isocratic HPLC method for the purity test of tablets containing metronidazole is described in British Pharmacopoeia (B.P.2011).

Tests of purity of the substance can be found in B.P.2001, Ph.Eur.7.0, and Polish Pharmacopoeia VIII. Neither tests of purity of chloroquinaldol tablets nor of the substance were found in the same documents, yet the corresponding TLC tests were inserted in an older source, Polish Pharmacopoeia VI. Only one paper dealing with chloroquinaldol was found, with the use of spectrophotometric method (1). On the other hand, many papers refer to metronidazole tested in medicinal products (1–8, 10–13, 17, 19) or biological material (9, 14–16, 18–21). A variety of methods has been applied in the tests of metronidazole: spectrophotometric method (2–6), NIR spectroscopy (7), voltammetric method (8, 9), HPTLC (10, 11), HPLC (12–16), UPLC-MS (17, 18), NMR (19) and LC-MS (20, 21). In a number of papers, metronidazole is reported to be measured in parallel to other drug substances.

No purity determinations of chlorquinaldol and metronidazole in medicinal products have been reported using a single chromatographic system (HPLC).

EXPERIMENTAL

Materials

- Drug product: GYNALGIN – vaginal tablets containing 100 mg of chlorquinaldol and 250 mg of metronidazole; manufactured by ICN Polfa Rzeszów S.A.; batch number: 010506; expiry date: May 2009.
- Reference standards: Chlorquinaldol manufactured by ICN Polfa Rzeszów S.A. batch number: C4011209; 2-aminophenol (impurity of chlor-

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quinaldol) manufactured by Acros Organics batch number: A0266307; metronidazole manufactured by Farchemia, batch number: 06548740; 2-methyl-5-nitroimidazole (impurity of metronidazole) manufactured by CRS BP, batch number: 2474.

Reagents and instrument

- High-purity HPLC reagents.
- Computer-controlled liquid chromatograph by Shimadzu, fitted with UV-VIS SPD-10AV*VP* detector, LC-10AT*VP* pump system, DGU-14A sample degassing unit, SCL-10A*VP* controller, and SIL-10AD*VP* automatic sample feeder.
- Chromatographic columns: Symmetry C8, 250 × 4.6 mm, by Waters, and Kromasil 100-5C8 250 × 4.6 mm, by AKZO NOBEL.

Standard solutions

A methanol standard solution (mix 0.5%) was prepared, 1 mL of which contained: 0.05 mg of 2-methyl-5-nitroimidazole, 0.05 mg of metronidazole (0.5% as compared to metronidazole concentration in the sample), 0.02 mg of 2-aminophenol, and 0.02 mg of chlorquinaldol (0.5% as compared to the concentration of chlorquinaldol in the sample).

Sample solutions

One gram of pulverized tablets was weighed and transferred to a 25 mL volumetric flask, then 15 mL of methanol was added to the sample. It was ultrasonicated for ca. 15 min then, agitated on a mechanical shaker for approx. 30 min, made up to volume with the solvent and filtered through a filter with 0.45 μm mesh size.

Table 1. Calibration curve parameters, correlation coefficients, detection limits (LOD) and quantitation limits (LOQ) of the analyzed substances.

Substance determined	Calibration curve parameters y = ax + b	LOD [μg/mL]	LOQ [μg/mL]
Metronidazole	a ± Δa = 45745 ± 857; S_a= 333 b ± Δb = 50136 ± 73949; S_b= 28768 S_y= 59567 r = 0.9999	4.30	13.02
Chlorquinaldol	a ± Δa = 134406 ± 4528; S_a= 1631 b ± Δb = -268279 ± 109842; S_b= 39562 S_y= 50294 r = 0.9997	1.23	3.74
2-Methyl-5-nitroimidazole	a ± Δa = 48750 ± 726; S_a= 261 b ± Δb = 18635 ± 44358; S_b= 15977 S_y= 20312 r = 0.9999	1.37	4.17
2-Aminophenol	a ± Δa = 13775 ± 278; S_a= 99 b ± Δb = 5052 ± 6446; S_b = 2322 S_y = 2954 r = 0.9999	0.71	2.14

a, b – regression coefficients; S_a, S_b – standard deviation of regression coefficients; S_y – standard error of the estimate; r – correlation coefficients.

Table 2. Results of system precision tests.

Substance determined	Average peak area [a.u.]	Standard deviation n = 6	RSD [%]
Metronidazole	1992389	14419	0.72
Chlorquinaldol	1880307	28223	1.50
2-Methyl-5-nitroimidazole	2070600	17141	0.83
2–Aminophenol	219760	2585	1.18

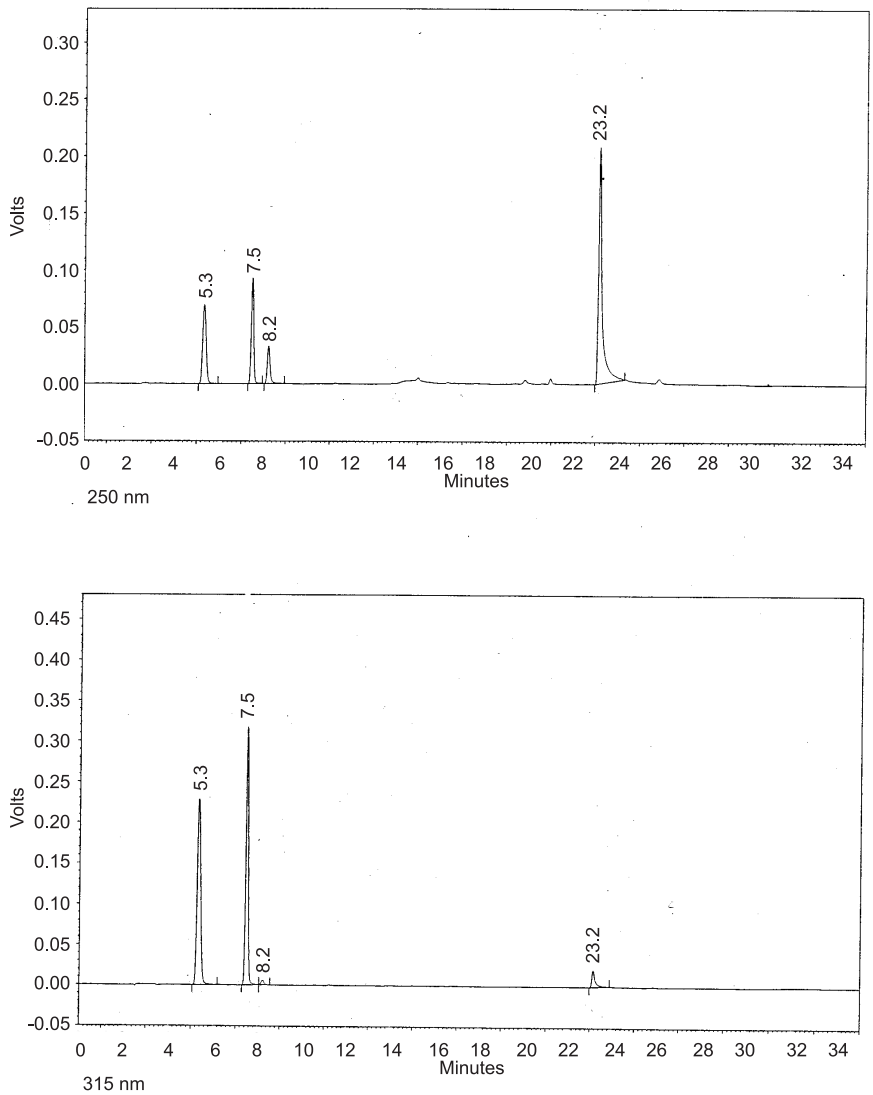

Figure 1. Chromatograms of the mixture of standards at 250 nm and 315 nm wavelengh. Retention times: 2-methyl-5-nitroimidazole 5.3 min.; metronidazole 7.5 min.; 2-aminophenol 8.2 min.; chlorquinaldol 23.2 min

The sample solution contained 10 mg/mL of metronidazole and 4 mg/mL of chlorquinaldol .

RESULTS AND DISCUSSION

HPLC method development

First, a specific HPLC system has been sought, which would provide sufficient separation and quantitative determination of impurities originating from metronidazole and chlorquinaldol. It was necessary to separate 2–aminophenol, 2-methyl-5-nitroimidazole, and unidentified impurities developed in the course of stress tests from the parent drug substances. as well as peaks originating from the solvent and placebo.

For the tests, the expired tablets were selected (1.5 years past their expiration date) with a new having more impurities.

A variety of gradient elution systems were tested, along with numerous modifications of mobile phases, flow rates and injection volumes. pH of the mobile phase influenced the 2-aminophenol peak location. The following chromatographic system was finally selected for further tests with the aid of gradient elution: Symmetry C8 column, 250 × 4.6 mm; 40°C; mobile phase A – a mixture of acetoni-

Table 3. Results of the determination of unknown impurities.

	Impurities originating from metronidazole [%]						Impurities originating from chlorquinaldol [%]			
	RT 3.8	RT 4.7	RT 9.1	RT 9.2	RT 10.0	Total	RT 24.4	RT 26.2	RT 28.8	Total
1	0.218	0.307	0.023	0.030	0.061	0.639	0.066	0.028	0.196	0.290
2	0.218	0.306	0.023	0.030	0.060	0.637	0.058	0.026	0.196	0.280
3	0.220	0.311	0.024	0.030	0.061	0.646	0.061	0.027	0.200	0.288
4	0.215	0.309	0.024	0.030	0.060	0.638	0.061	0.028	0.196	0.285
5	0.219	0.308	0.023	0.030	0.061	0.641	0.064	0.027	0.197	0.288
6	0.213	0.308	0.023	0.030	0.060	0.634	0.054	0.023	0.190	0.267
Mean	**0.216**	**0.307**	**0.023**	**0.030**	**0.060**	**0.639**	**0.060**	**0.025**	**0.193**	**0.283**
SD	0.0038	0.0004	0.0002	0.0001	0.0007	0.004	0.0082	0.032	0.0036	0.01
%RSD	1.75	0.14	0.74	0.28	1.21	0.64	13.75	12.59	1.89	3.03

RT – retention time

trile and buffer, pH 5.5 (10 : 90, v/v); mobile phase B – a mixture of acetonitrile and buffer, pH 5.5 (75 : 25, v/v); buffer, pH 5.5 – a mixture of 1,000 mL of water and 1 mL of 85% H_3PO_4 adjusted to pH 5.5 with 10% NaOH solution.

Gradient elution:

Time [min]	Mobile phase B [%]
0 – 20	0 → 100
20 – 25	100
25 – 27	100 → 0
27 – 35	0

Mobile phase flow rate was 1.2 mL/min, injection volume 20 μL, and detection wavelengths: 250 nm (to determine 2-aminophenol and unidentified impurities originating from chlorquinaldol) and 315 nm (to determine 2-methyl-5-nitroimidazole and other unidentified impurities originating from metronidazole) .

The following system performance parameters were selected: symmetry coefficient ($A_{10\%}$) of metronidazole peak, which ought to be not higher than 1.5, and resolution (R_s) between the peaks for 2-aminophenol and metronidazole, not lower than 3.0. The parameters were measured with the mix 0.5% solution.

Validation of the method

Specificity

In order to examine the method specificity, solvent, solution of excipients (placebo), solutions of identified impurities (2-methyl-5-nitroimidazole and 2–aminophenol) and unidentified impurities (obtained in the course of stress tests) along with solutions of standard drug substances were injected onto the column. The recorded chromatograms revealed that the peaks originating from the solvent, drug substances and excipients did not interfere with the peaks originating from impurities.

Linearity

A dependence was examined between the peak areas on the chromatogram and the concentrations of specific substances and was found to be linear within the following concentrations:

0.01 mg/mL – 0.1 mg/mL for 2-methyl-5-nitroimidazole;

0.004 mg/mL – 0.04 mg/mL for 2-aminophenol;

0.01 mg/mL – 0.1 mg/mL for metronidazole;

0.004 mg/mL – 0.04 mg/mL for chlorquinaldol.

Parameters of the calibration curves along with the calculated correlation coefficients are presented in Table 1.

Table 4. Results of recovery.

Related substance	Amount added [mg/mL]	Amound found [mg/mL]	Recovery [%]	
2-Methyl-5-nitroimidazole	0.0242 0.0242 0.0484 0.0484 0.0792 0.0792	0.0239 0.0241 0.0481 0.0481 0.0788 0.0790	98.80 99.57 99.44 99.44 99.50 99.77	Mean: 99.29 ± 0.26 SD: 0.33 %RSD: 0.33
2–Aminophenol	0.0125 0.0125 0.0213 0.0213 0.0330 0.0330	0.0105 0.0108 0.0175 0.0177 0.0291 0.0283	83.49 85.96 82.00 82.71 83.44 85.72	Mean: 83.89 ± 1.29 SD: 1.61 %RSD: 1.92

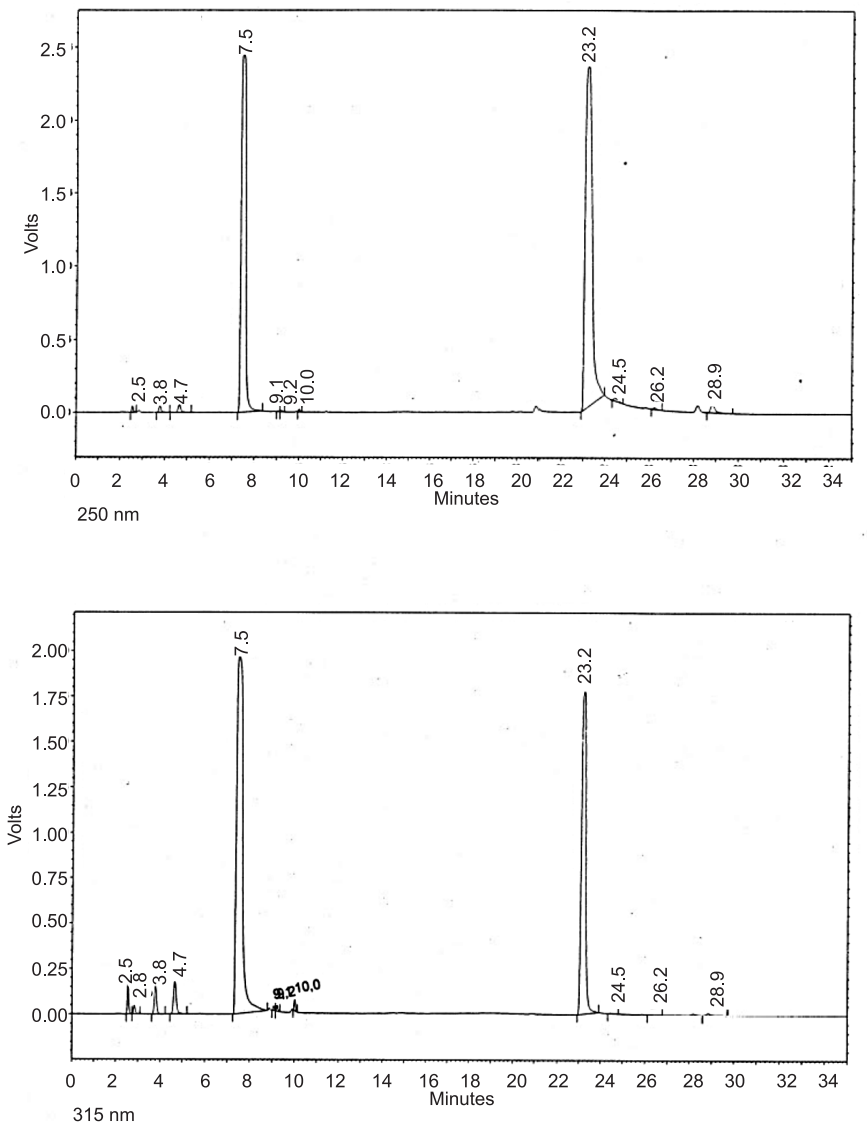

Figure 2. Chromatograms of Gynalgin vaginal tablets at 250 nm and 315 nm wavelength. Retention times: metronidazole 7.5 min.; 2-chlorquinaldol 23.2 min

Table 5. Robustness of the proposed HPLC method.

		Retention time (min)				Symmetry coefficient ($A_{10\%}$)				Resolution (R_S)			
		2-Methyl-5-nitroimidazole	Metronidazole	2-Aminophenol	Chlorquinaldol	2-Methyl-5-nitroimidazole	Metronidazole	2-Aminophenol	Chlorquinaldol	2-Methyl-5-nitroimidazole	Metronidazole	2-Aminophenol	Chlorquinaldol
Flow rate	1.1 mL/min	5.86	8.18	8.89	23.98	1.18	1.06	1.17	1.71	–	8.84	3.34	–
	1.2 mL/min	**5.41**	**7.57**	**8.27**	**23.21**	**1.18**	**1.06**	**1.19**	**1.82**	–	**8.92**	**3.42**	–
	1.3 mL/min	4.99	7.05	7.73	22.55	1.16	1.06	1.19	1.90	–	8.80	3.38	–
Temperature	35°C	5.70	7.82	8.48	23.51	1.12	1.07	1.17	1.64	–	8.79	3.37	–
	40°C	**5.42**	**7.58**	**8.29**	**23.24**	**1.13**	**1.05**	**1.18**	**1.76**	–	**8.73**	**3.36**	–
	45°C	5.20	7.38	8.05	23.0	1.12	1.05	1.12	1.76	–	8.89	3.09	–
pH in a buffer added to mobile phase	pH 5.3	4.84	6.87	7.70	23.10	1.18	1.08	1.20	1.91	–	8.44	3.62	–
	pH 5.5	**4.86**	**6.91**	**7.80**	**22.98**	**1.17**	**1.08**	**1.20**	**1.80**	–	**8.61**	**3.98**	–
	pH 5.7	4.83	6.87	7.81	23.05	1.14	1.06	1.19	1.90	–	8.60	4.20	–
Different columns	**Symmetry C8**	**5.44**	**7.54**	**8.36**	**23.44**	**1.17**	**1.07**	**1.08**	**1.09**	–	**8.93**	**4.15**	–
	Kromasil 100-5C8	5.16	7.3	8.11	23.26	1.15	1.05	1.19	1.79	–	8.73	3.75	–

Limit of detection and limit of quantification

Limit of detection (LOD) and limit of quantification (LOQ) were calculated on the basis of the relevant calibration curve (straight line) parameters, according to the following formulas: LOD = 3.3×Sy/a and LOQ = 10×Sy/a, where Sy – standard error of estimation, a – slope of the straight line. Results are presented in Table 1.

Precision of the chromatographic system

In order to estimate the system precision, the 0.5% mix standard solution was analyzed six times. RSD = 5% was chosen as an acceptance criterion, where RSD-relative standard deviation of the six peaks of two parent substances and known impurities. The results are presented in Table 2.

Precision of the method

In order to assess the method precision, the statistical analysis included the results from the six independent determinations of all impurities contents. RSD = 15% was used as an acceptance criterion. The results are presented in Table 3.

Accuracy

Specific quantities of 2-methyl-5-nitroimidazole and 2-aminophenol were added to weighed samples of the preparation to correspond to 50%, 100% and 150% of the permitted limit. The samples were dissolved in methanol, filtered and added to a chromatographic column (20 µL each). 80% – 120% recovery ratio was used as an acceptance criterion. The results are shown in Table 4.

Robustness of the method

The effect of the mobile phase flow ratio (± 0.1 mL/min), column temperature (± 5°C), pH of water contained in the mobile phase (± 0.2) and column type on the retention times, symmetry coefficients ($A_{10\%}$) and resolution coefficients (R_S) were evaluated for the standards 0.5% mix solution. The results are presented in Table 5.

Stability of solutions

Standard (0.5% mix*)* and sample solution were stored at ambient temperature and in refrigerator (4–6°C) over 24 h. Solutions were analyzed at 0 and 24 h. The results were evaluated as the percent difference of peak area of each impurity from that for the freshly prepared solutions. Less than 5% difference was observed which demonstrates that the standard and sample preparations were stable for up to 24 h.

Determination of the content of impurities

Twenty microliters of each of the prepared solutions: mixture of standards (mix 0.5%), samples and placebo was added to the chromatographic column. Chromatograms of the standard solutions are shown in Figure 1, and of the sample solutions – in Figure 2. It can be seen that no identified impurities (2-methyl-5-nitroimidazole and 2-aminophenol) were detected in the sample solutions.

Of the unknown impurities, those originating from metronidazole were tested at 315 nm wavelength, and those originating from chlorquinaldol were examined at 250 nm wavelength. Assay of the unknown impurities was based on comparison of their peak areas with those of diluted drug substances contained in mix 0.5% solution. Content of individual impurities arising from metronidazole was ranged from 0.02% to 0.3%; total content – 0.6%, whereas impurities arising from chlorquinaldol ranged from 0.02 to 0.2%, total content 0.3.%. All impurities amounted to 0,9%.The results are shown in Table 3.

CONCLUSIONS

Results of the study prove that the determination of impurities in Gynalgin two-component preparation containing metronidazole and chlorquinaldol can be reliably performed with the designed HPLC method. The method was shown to comply with the acceptance criteria, including sufficient separation coefficients (R_S) between the closest peaks. Its specificity was confirmed as the peaks originating from the solvent, excipients, known impurities and drug substances did not interfere. Calibration curves of the two parent substances and their two known impurities were linear (correlation coefficients higher than 0.999). Precision of the method of impurity content determination was characterized by RSD below 14%. Recovery of impurities 2-methyl-5-nitroimidazole was 98.8–99.8% and that of impurities 2-aminophenol was 82.0–86.0%. Solutions of standards and samples were shown to be stable for 24 h at ambient temperature and at 4–6°C temperature (in refrigerator). Minor variations in the chromatography conditions were shown to have no effect on the performance of the method, thus it can be reckoned as a robust one It also allows for quantification of impurities within a relatively short period of time (35 min), and it is another advantage of the proposed method.

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