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DEVELOPMENT OF A METHOD FOR ASSESSING THE STABILITY OF AFOBAZOLE BY HPLC

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The aim of the present work was to develop a method for assaying afobazole in substance and medicinal formulations for assessment of the contents of both technical contaminants and likely degradation products. Studies were performed using HPLC on a liquid chromatogram with a spectrophotometric detector in the following conditions: a Phenomenex column (250 × 4.6 mm, sorbent Luna C18(2), 5 μm); mobile phase: acetonitrile, methanol and 0.02 M potassium hydrogen phosphate pH 7.3 solution at a ratio of 100:100:240 (by volume). A method was developed allowing separation of afobazole and its initial and intermediate synthesis products, oxidation products, and other afobazole degradation products produced in acidic and alkaline media and on oxidation and exposure to sunlight. This method yields data on the stability of the substance and medicinal formulations both during preparation and on storage.

Key words: Afobazole, stability, high-performance liquid chromatography.

There is an increasing preference for high-performance liquid chromatography (HPLC) in the development of methods for the qualitative and quantitative analysis of medicines. However, there is no doubt of the need for additional investigations at the stage of developing methods allowing assessment of selectivity for not only technical contaminants, but also degradation products of medicines. Interest in this problem is evidenced by numerous studies addressing the development and validation of analytical methods using so-called "stress tests." [1 – 3], whose use is recommended by the International Conference on the Harmonization of Technical Requirements for the Registration of Medicines for Human Use [4, 5].

Studies at the V. V. Zakusov Science Research Institute of Pharmacology, Russian Academy of Medical Sciences, have developed the new selective anxiolytic afobazole, or 5-ethoxy-2-[2-(morpholino)ethylthio]benzimidazole dihydrochloride (I, Fig. 1) [6].

The aim of the present work was to study and develop an HPLC method for assay of I in substance and medicinal formulations allowing evaluation of contents of both technical contaminants and likely degradation products.

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EXPERIMENTAL SECTION

Studies were performed using samples of substance I, tablets of I, 5 mg, and solution of I for injection, 10 mg/ml.

Marker substances were initial and intermediate products of synthesis of I: phenacetin (VI), 3-nitro-4-aminophenetole (VII), 3,4-diaminophenetole (VIII), and 5-ethoxy-2-mercaptobenzimidazole (IX), the intermediate synthesis product 5-ethoxy-1(3)-(2-morpholineoethyl)-2-[2-(morpholino-ethyl thio]benzimidazole (X), and suggested degradation products of I: 5-hydroxy-2-[2-(morpholino)ethylthio]benzimidazole (II), afobazole sulfate (III), 5-ethoxy-6-ketobenzimidazole dihydrochloride (IV), and 5-ethoxy-2-[2-hydroxy)ethylthio]benzimidazole (V) (see Fig. 2).

Studies of the chromatographic mobility of I and marker substances by HPLC were performed using Shimadzu (Japan) and Beckman (USA) liquid chromatography systems fitted with variable-wavelength spectrophotometric detectors. Study compounds were separated on Luna C18 (2) columns (length 250 mm, internal diameter 4.6 mm, sorbent C_{18} with particle size 5 μm).

The mobile phase was a mixture of acetonitrile, methanol, and 0.02 M potassium hydrogen phosphate pH 7.3 (100:100:240 by volume); the elution regime was isocratic, the mobile phase flow rate was 1 ml/min, the column was at

room temperature, and the analytical wavelength was 302 nm.

Study compounds were dissolved in mobile phase or one of its components with subsequent dilution of the resulting solution with mobile phase; sample volume was 20 μl.

RESULTS AND DISCUSSION

Studies of the chromatographic mobility of I and suggested contaminants were performed using a mobile phase selected to give complete separation of the compounds of interest: a mixture of acetonitrile, methanol, and 0.02 M potassium hydrogen phosphate solution pH 7.3 (100:100:240 by volume).

The analytical wavelength was selected on the basis of the UV spectra of solutions of the study compounds in the mobile phase. The UV spectra of most compounds had maxima in the ranges 200 – 220 nm and 280 – 330 nm. Compound I and contaminants were detected at 302 nm, as this corresponded to the position of the peak of solutions of I, the solution of the intermediate product of the last stage of the synthesis (IX), and all suggested degradation products (II – V).

Figure 3 shows a chromatogram of a model mixture of I and marker substances at a level of about 0.5% of that of compound I. Retention times, detection limits at 302 nm, and separation parameters are shown in Table 1.

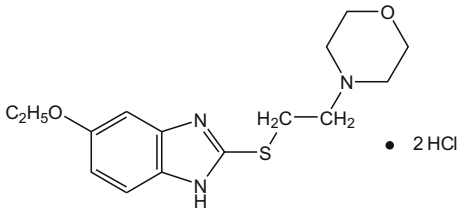

Fig. 1. Structural formula of I.

A linear relationship between peak area and the concentrations of solutions of I was seen over the concentration range 0.0001 – 0.4 mg/ml, with a correlation coefficient of $r = 0.999$ (see Fig. 4). Linear relationships between peak areas and the concentrations of marker solutions were seen over the concentration range 0.005 – 0.1 mg/ml, with correlation coefficients of at least 0.999 (see Fig. 5).

Given the detection limits for contaminants, a working concentration of I of 1 mg/ml was selected. This concentration allowed detection of known contaminants at levels of 0.1% and less of that of I in test solutions.

Analysis of model solutions of I and contaminants was used to calculate correction coefficients for assessment of concentrations of contaminants in I. Correction coefficients for compounds V, III, IV, and X were close to unity (from 0.98 to 1.07); the correction coefficient of IX was 0.48, that for II was 0.79, that for VII was 2.32, that for VIII was 2.50, and that for VI was 8.04.

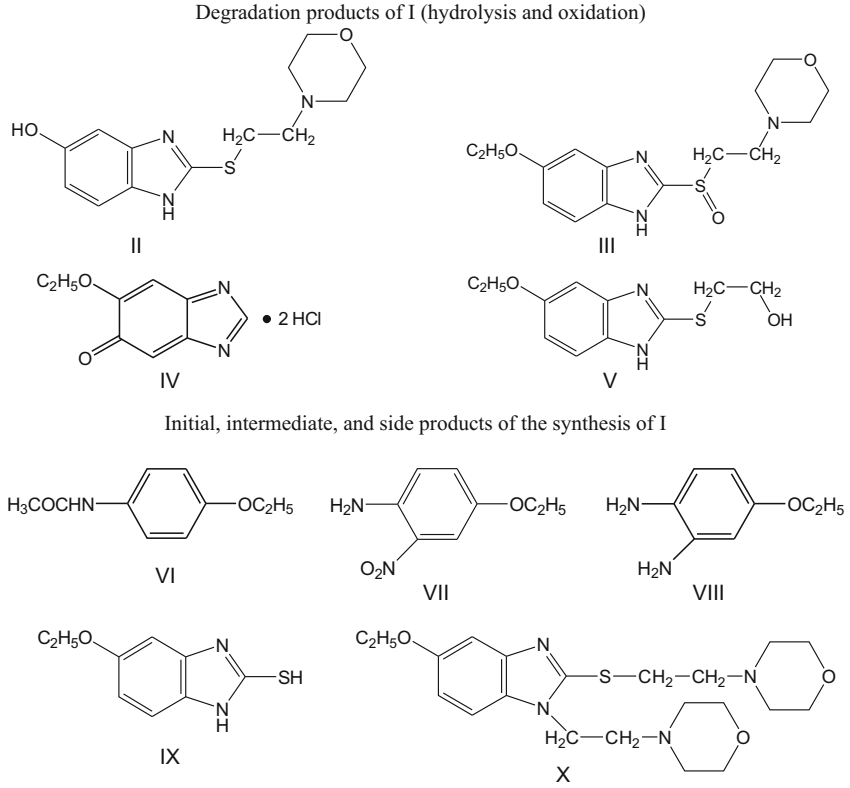

Fig. 2. Initial, intermediate, and side products of the synthesis of I and is degradation products.

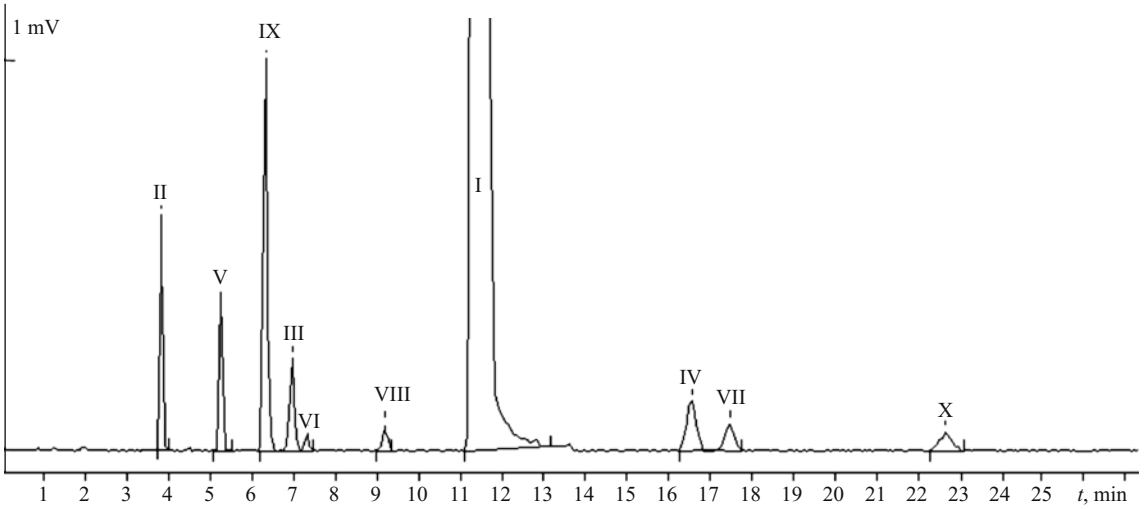

Fig. 3. Chromatogram of a model mixture of I and marker compounds.

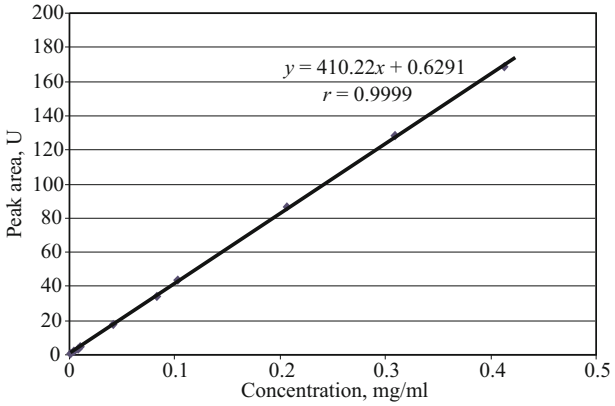

Fig. 4. Relationship between peak areas and concentrations of I solutions.

The selectivity of the method in relation to the degradation products of I was studied by preparing solutions of I in water, in 0.1 M hydrochloric acid and sodium hydroxide and in 3% hydrogen peroxide. The concentration of I in these solutions was 10 mg/ml.

Solutions were placed in an incubator at 40°C for periods ranging from 4 (samples in 3% hydrogen peroxide) to 28 days (samples in water, 0.1 M hydrochloric acid and sodium hydroxide) with periodic monitoring of external appearance and chromatographic purity. Solutions of I in water were also placed in direct sunlight for two months.

At the end of the storage period, 1 ml of each solution (apart from the solution in 3% hydrogen peroxide) was placed in a 10-ml measuring flask and the volume was made up to the mark with mobile phase and mixed (the theoretical concentration of I was 1 mg/ml). Control solutions containing 1 ml of the solvents corresponding to the study samples in 10 ml of mobile phase were prepared and analyzed in parallel.

The solution of I in 3% hydrogen peroxide (1 ml) was evaporated in vacuo on a rotary evaporator at room temperature and the residue was dissolved in 10 ml of mobile phase to obtain afobazole solution with a theoretical concentration of 1 mg/ml. The control solution was prepared in the same way, using 1 ml of 3% hydrogen peroxide. Peaks common to the histograms of the test and control solutions were ignored.

Contaminants in study samples were identified by agreement between their relative retention times (relative to I) and the relative retention times of marker substances II-X by addition of known contaminants to test solutions, as well as from the coincidence of retention times when the ratio of mobile phase components was changed.

Contaminant contents were analyzed by preparing a solution of the working standard sample of I with a concentration of 0.005 mg/ml; contents of known contaminants were calculated using correction coefficients.

These studies showed that degradation of I in acidic and alkaline media was insignificant and mainly formed oxidation products III and V. At four weeks in 0.1 M hydrochloric acid, the main product was contaminant V (0.05%); the main product in 0.1 M sodium hydroxide was contaminant III (about 0.3%), along with a further six unidentified contaminants with a total content of less than 0.4%. Aqueous solutions held for two months showed no change in the chromatographic purity of I.

Hydrogen peroxide (3%) produced significantly faster degradation of I. By one day, the total contaminant content was about 8% and the solution was found to contain both the known degradation products of I, i.e., contaminants III (3.0%), V (1.49%), and II (0.31%), and unidentified degradation products with relative retention times (relative to I) of 0.20 (0.31%), 0.21 (0.64%), 0.24 (0.18%), 0.26 (0.12%), 0.30 (0.04%), and 0.41 (1.79%). The chromatogram of the test solution is shown in Fig. 6.

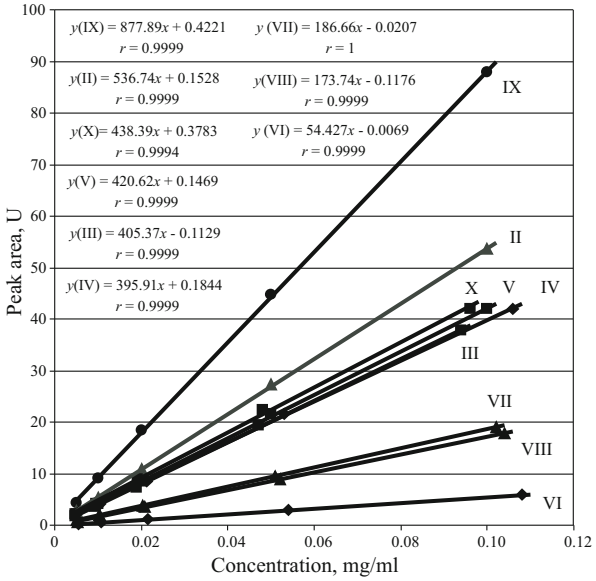

Fig. 5. Relationships between peak areas and concentrations of contaminants of I.

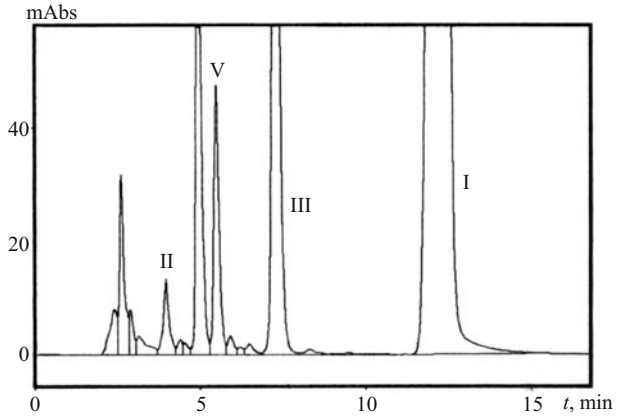

Fig. 6. Chromatogram of solution of I in 3% hydrogen peroxide after storage for one day at 40°C.

Solutions of I exposed to sunlight for four weeks showed 11 contaminants with a total content of about 30%. These included the known oxidation products of I: contaminants IX (18.3%), II (0.59%), V (0.25%), and IV (0.44%). The solutions contained contaminants with relative retention times of 0.26 (0.53%), 0.72 (0.23%), 0.87 (1.97%), 1.34 (1.87%), 1.64 (5.59%), and 2.07 (0.22%), as well as 0.22, 0.36, 0.42, 0.50, and 0.80 (each less than 0.2%). The chromatogram of the test solution is shown in Fig. 7. At eight weeks, the total content of contaminants increased to 43% and the content of contaminants with retention times coinciding with the known degradation products of I increased almost two-fold.

These data show that separation in these chromatography conditions discriminated I, five of its initial, intermediate, and side products of synthesis, four oxidation products, and a series of other contaminants which could not be identified. The nature of the contaminants in test solutions of I provides evidence that the main pathway of degradation of I in aqueous medium is oxidation.

The suitability of the chromatography system was verified using solutions of I and IX with component concentrations of 0.05 mg/ml. Suitability was evaluated in terms of the efficiency of the chromatography column, the asymmetry coefficient of the compound I peak, the coefficient of separation (resolution) of the peaks for I and 5-ethoxy-2-mercaptobenzimidazole, and the relative standard deviation calculated for the peaks of compound I in five parallel measurements. Table 2 shows the extreme values for actual determinations of measures of the suitability of the chromatography system obtained at different time points.

These conditions were used for analysis of serial samples of substance I, 5-mg tablets of I, and 10-mg/ml solutions of I for injection, with one change: despite the fact that the specific absorption of solutions of I and marker substances differed, complexity in the method was avoided by not using the coefficients, but by assessing the contents of individual contaminants by comparing the areas of contaminants and the working standard sample at a concentration of 0.005 mg/ml in solution.

The test solution for analysis of substance was prepared as follows: 0.01 g (accurately weighed) of substance was

TABLE 1. Retention Times, Detection Limits, and Separation Parameters of I and its Likely Contaminants.

Compound	Retention time (t_{ret}), min	Relative retention time (t_{rel})	Detection limit, μg (302 nm)	R_s for neighboring pairs
II	3.81 ± 0.08	0.33 ± 0.005	0.0020	–
V	5.23 ± 0.10	0.45 ± 0.008	0.0040	4.73
IX	6.32 ± 0.10	0.55 ± 0.007	0.0015	3.11
III	7.01 ± 0.11	0.61 ± 0.008	0.0020	1.73
VI	7.30 ± 0.07	0.63 ± 0.008	0.0300	1.11
VIII	9.20 ± 0.06	0.81 ± 0.010	0.0120	7.62
I	11.51 ± 0.23	1.00	0.0030	1.93
IV	16.62 ± 0.12	1.45 ± 0.010	0.0035	3.01
VII	17.53 ± 0.10	1.53 ± 0.012	0.0070	1.33
X	22.55 ± 0.10	1.98 ± 0.007	0.0035	6.69

TABLE 2. Suitability Parameters of the Chromatography System for Analysis of I.

Chromatography system suitability parameters			
N (afobazole)	Q (afobazole)	R_s (afobazole)	RSD (afobazole)
4465 ± 427	1.2 ± 0.1	6.7 ± 0.2	Not greater than 1.4%
Acceptance criterion			
At least 4000	Not greater than 1.5	At least 6.0	Not greater than 2%

TABLE 3. Contents of Contaminants in Substance and Medicinal Formulations of I.

Medicinal formulation	Extremes of contents of individual contaminants, %						Total contaminants, %
	IX	Oxidation products of I		Unidentified contaminants			
		II	V	t_{rel} 0.27	t_{rel} 0.62	t_{rel} 0.72	
Substance	0 – 0.1	0 – 0.06	–	0 – 0.04	–	0 – 0.07	0 – 0.17
Tablets, 5 mg	0 – 0.1	0 – 0.04	–	0 – 0.08	0.09	0 – 0.14	0.04 – 0.34
Solution for injection, 10 mg/ml	0 – 0.30	0 – 0.07	0.01 – 0.25	0 – 0.04	–	0 – 0.11	0.04 – 0.64

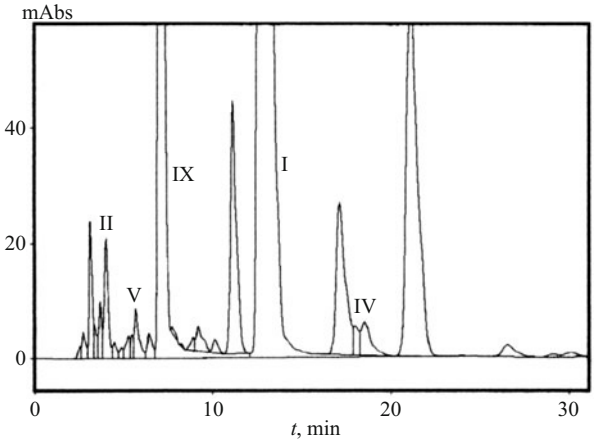

Fig. 7. Chromatogram of solution I in water after four weeks of storage in sunlight.

placed in a 10-ml measuring flask, dissolved in a sufficient quantity of mobile phase, and made up to the mark with mobile phase and mixed.

For analysis of tablets, accurately weighed powder from ground tablets equivalent to 0.025 g of I was placed in a 25-ml measuring flask, shaken with 15 ml of mobile phase for 10 min, and made up to the mark with mobile phase, mixed, and filtered through a Millipore filter with a pore diameter of 0.45 μm.

Test solution was prepared for analysis of solution of I for injection by placing 1 ml of solution in a 10-ml measuring flask and making the volume up to the mark with mobile phase and mixing.

Analysis of medicinal formulations demonstrated the absence of peaks due to leached excipients.

Samples of I substance contained contaminants IX and II, along with two unidentified contaminants with relative retention times (relative to I) of about 0.27 and 0.72. The content of any single contaminant was no greater than 0.1% and the total contaminant content was no greater than 0.2% (Table 3).

Samples of afobazole tablets contained contaminants IX and II, along with three unidentified contaminants with relative retention times of 0.27, 0.62, and 0.72, none at a level exceeding 0.15%. The total contaminant content was no greater than 0.4% (Table 3).

Analysis of serial samples of I solution for injection showed that samples contained contaminants IX, II, and V, along with unidentified contaminants with relative retention times of about 0.27 and 0.72. Levels of individual contaminants were no greater than 0.2% and total contaminant content was no greater than 0.7% (Table 3).

These results indicate that samples of substance and medicinal formulations of I contained, apart from technical contaminant IX, oxidation products II and V, and three unidentified contaminants.

Thus, the method developed here, which allows separation of I and its most likely contaminants, i.e., the initial and intermediate synthesis products and the suggested oxidation products when present in substance, tablets, and solution for injection. The method also allows detection of other degradation products of I formed on exposure to aggressive environmental factors, and thus provides data on the stability of the substance and medicinal formulations both at the preparation stage and after storage.

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