# STRUCTURE OF CHEMICAL COMPOUNDS, METHODS OF ANALYSIS AND PROCESS CONTROL

## DEVELOPING ANALYTICAL METHODS FOR AFOBAZOLE IN INJECTION FORMS

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This investigation was aimed at elaborating procedures for the pharmaceutical analysis of the parenteral dosage form of afobazole, a novel domestic drug with anxiolytic and neuroprotective action. The physicochemical properties of afobazole parenteral dosage form have been studied. Methods for the determination of impurities (TLC, HPLC) and identification and assay of the drug (UV spectrophotometry, HPLC) have been developed.

**Key words:** afobazole, parenteral dosage form, pharmaceutical analysis, thin-layer chromatography, high performance liquid chromatography.

Afobazol (**I**), 5-ethoxy-2-[2-(morpholino)ethylthio]-benzimiadzole hydrochloride (Fig. 1), is a new selective anxiolytic that was developed at Zakusov Institute of Pharmacology, Russian Academy of Medical Sciences (RAMS) [1]. Pharmacological trials of **I** showed that it exhibits neuroprotective action in addition to anxiolytic activity [2]. The manifestation of neuroprotective properties, in turn, made it possible to administer **I** in combined therapy for acute disruptions of brain blood flow, owing to which it was necessary to develop a parenteral form of the drug.

A parenteral form of **I** as an aqueous isotonic solution with a concentration of 10 mg/mL was developed at the Institute of Pharmacology, RAMS. This investigation was aimed at studying the physicochemical properties and developing a method for pharmaceutical analysis of the parenteral solution of **I**.

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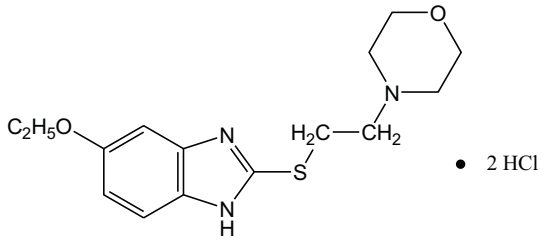


**Fig. 1.** Structural formula of **I**.

## EXPERIMENTAL PART

Studies were carried out on serial samples of the parenteral solution of **I** (10 mg/mL). The physicochemical properties of the preparation relating to the pharmacopoeial quality indicators were determined. Methods for determining the authenticity, purity, and quantitative determination of **I** in the parenteral form were developed.

UV spectrophotometry and thin-layer (TLC) and high performance liquid chromatography (HPLC) were used for the studies.

UV spectra for determining the authenticity and absorption coefficients of the solutions for quantitative determination were obtained on a UV-1700 spectrophotometer (Shimadzu, Japan).

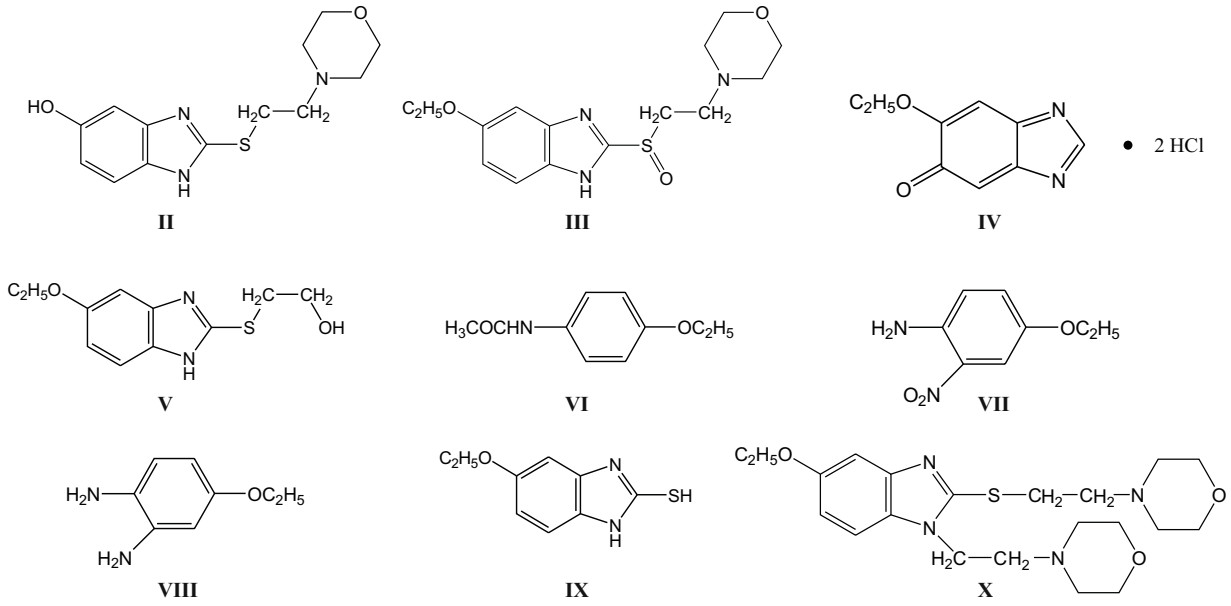


**Fig. 2.** Probable hydrolysis and oxidation products and intermediates in the synthesis of **I**.

The purity of the solutions of **I** was determined using TLC and HPLC.

TLC was performed on Kieselgel 60 $F_{254}$ plates (Merck). Chromatography was carried out using acetone:hexane:conc. $NH_4OH$ (20:20:0.5) with detection by UV light (254 nm) and iodine vapor.

The chromatographic mobility of solutions of **I** was studied in order to determine the purity and quantitative content using HPLC in Shimadzu (Japan) and Beckman (USA) liquid chromatographs equipped with variable-wavelength spectrophotometric detectors.

The separations were carried out in Diasphere-110-$C_{18}$ (150 mm × 4.0 mm, $C_{18}$ sorbent, 7 μm) [3] and Luna $C_{18}$ (2) (250 mm × 4.6 mm, $C_{18}$ sorbent, 5 μm) steel columns.

## RESULTS AND DISCUSSION

All samples of the parenteral form of **I** appeared as a colorless or slightly colored liquid. The solutions were transparent. The coloration did not exceed that of standard 5b. The pH value fell in the range 2.0 – 3.0.

The UV spectrum of solutions of **I** diluted with HCl solution (0.01 M) to a concentration of 0.015 mg/mL exhibited in the wavelength range 210 – 350 nm a characteristic maximum at 302.0 ± 2 nm (actual data for the position of the maximum in spectra of solutions of **I** were 302.30 ± 0.64 nm).

The active ingredient and its impurities were extracted from the aqueous solution by $CHCl_3$:$Et_2NH$ (9:1) with equal volumes of the aqueous and organic phases in order to analyze the purity of the parenteral solution of **I**. A volume of the $CHCl_3$ extract equivalent to 100 μg of **I** was placed at the origin. Bands were detected using UV light and iodine vapor.

The content of impurities was estimated visually by comparing the size and intensity of bands of impurities and solutions of a working standard of **I** placed on the chromatographic plate in an amount equivalent to 0.2 μg (0.2%) and 0.1 μg (0.1%) of **I**.

The suitability of the chromatography system was evaluated using the $R_s$ value of **I** ($R_f$ ~ 0.27) and 5-ethoxy-2-mercaptobenzimidazole ($R_f$ ~ 0.54), which was in the range 2.0 ± 0.3.

Analysis of released solutions using the method described above showed the presence of up to three unidentified impurities with $R_f$ values about 0.18, 0.36, and 0.42 ($R_s$ 0.67, 1.33, and 1.56, respectively). The content of each pure impurity was less than 0.2%. An impurity of 5-ethoxy-2-mercaptobenzimidazole, the content of which was less than 0.2%, was observed in several samples. Like in the drug sub-

**TABLE 1.** Retention Times, Detection Limits, and Separation Parameters of **I** and Its Probable Impurities

| Compound | Retention time ($t_r$), min | Relative retention time ($t_{rel}$) | Detection limit, μg (302 nm) | $R_s$ of 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 | 1.000 | 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 |

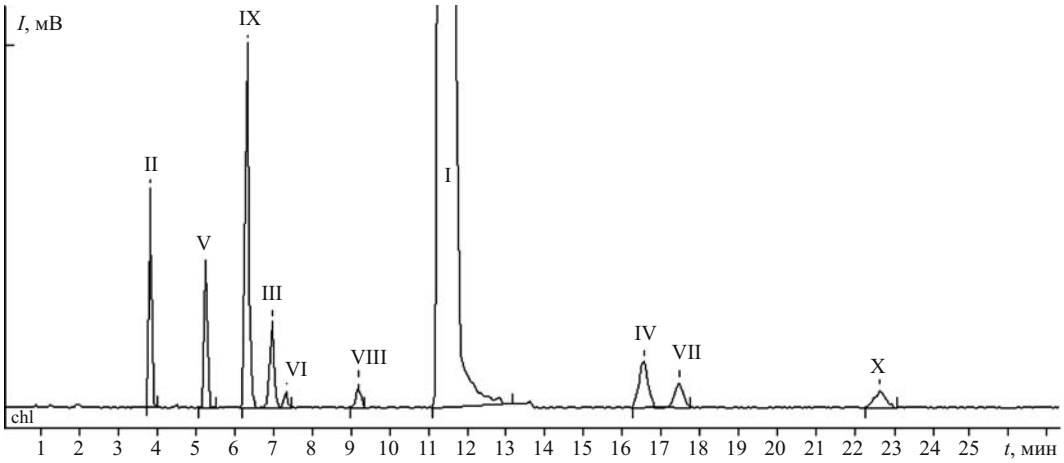


**Fig. 3.** Chromatogram of a model mixture of **I** and standards.

stance, an impurity of a synthetic intermediate of **I**, chloroethylmorpholine, was not detected in the parenteral solutions. The total impurities in each sample were less than 0.4%.

Chromatographic analysis of **I** by HPLC was performed under conditions that were used previously for the drug substance [3] using a Diasphere-110-$C_{18}$ column (150 mm × 4.0 mm, $C_{18}$ sorbent, 7 μm). The mobile phase was a mixture of eluents including MeOH:$H_2O$ (pH 7.6, 1:1, v/v). The pH value of the water was adjusted to 7.6 using NaOH solution (0.2 M). Isocratic elution at flow rate 1 mL/min was used. The analytical wavelength was 302 nm. The sample volume was 20 μL.

The drug (1 mL) was placed into a 10-mL volumetric flask, adjusted to the mark using mobile phase, and stirred (solution concentration 1 mg/mL). The resulting solutions were chromatographed at least five times for each solution.

The analysis time under the aforementioned conditions was about 25 – 30 min. The peak width of **I** at a height from the baseline that was 5% of the total height was about 3.0 – 3.5 min. The retention time was about 11 min. The efficiency of the chromatography column that was calculated using the peak of **I** was less than 1200 theoretical plates. In some instances the afobazole peak changed shape. This interfered with its quantitative determination. Adding phosphate buffer (0.02 M) to the mobile phase produced a narrower peak for **I** (the width at a height from the baseline of 5% was about 1 min) and improved its shape. Furthermore, it lowered the pH value of the aqueous phase without changing significantly the retention time of **I** and the process impurities.

Impurities that were absent in samples of the drug substance were detected using the developed method during the analysis of the purity of parenteral solutions of **I**. These were an impurity of 5-ethoxy-2-mercaptobenzimidazole and from one to four unidentified impurities.

We assumed that the observed impurities were oxidation products of **I** and made attempts to identify them. For this, several probable hydrolysis and oxidation products of **I** were synthesized. These were 5-hydroxy-2-[2-(morpholino)ethyl-

**TABLE 2.** Quantitative Determination of Model Solutions of **I** Using UV Spectrophotometry

| Model solution No. | Added (% of reference solution concentration) ($X_i$, %) | Found (% of reference solution concentration) ($Y_i$, %) | Found (% of added) $Z_i = 100\cdot(Y_i/X_i)$, % |
|---|---|---|---|
| 1 | 80.25 | 80.13 | 99.85 |
| 2 | 85.66 | 85.63 | 99.96 |
| 3 | 90.89 | 91.12 | 100.25 |
| 4 | 94.95 | 94.59 | 99.62 |
| 5 | 101.01 | 100.97 | 99.96 |
| 6 | 105.32 | 105.73 | 100.39 |
| 7 | 109.74 | 110.03 | 100.26 |
| 8 | 115.14 | 114.96 | 99.84 |
| 9 | 120.37 | 120.96 | 100.49 |
| Average $Z$, % | | | 100.07 |
| Standard deviation $S_Z$, % | | | 0.29 |
| Linear regression ($y = bx + a$) parameters | | | |
| a | $S_a$ | $b$ | $S_b$ | $r$ |
| – 1.037 | 3.4 | 1.011 | 0.034 | 0.999 |

**TABLE 3.** Quantitative Determination of Serial Samples of Parenteral Solution of **I** Using UV Spectrophotometry

| Series No. | Quantitative content, mg/mL | Metrological characteristics ($P$ = 95%, $n$ = 5) | | |
|---|---|---|---|---|
| | | $S$ | $\Delta\bar{X}$ | $\bar{\varepsilon}$, % |
| 1 | 10.53 | 0.072 | 0.090 | 0.85 |
| 2 | 9.94 | 0.049 | 0.061 | 0.61 |
| 3 | 10.31 | 0.053 | 0.066 | 0.64 |
| 4 | 9.83 | 0.091 | 0.113 | 1.15 |
| 5 | 10.12 | 0.038 | 0.047 | 0.46 |

thio]benzimidazole (**II**), the sulfoxide of **I** (**III**), 5-ethoxy-6-ketobenzimidazole dihydrochloride (**IV**), and 5-ethoxy-2-[2-(hydroxyl)ethylthio]benzimidazole (**V**) (Fig. 2). The compounds were used as standards for chromatographic analysis of the parenteral solutions of **I**.

Thus, synthetic intermediates of **I** that were available to us as standards were phenacetin (**VI**), 3-nitro-4-aminophenetole (**VII**), 3,4-diaminophenetole (**VIII**), 5-ethoxy-2-mercaptobenzimidazole (**IX**), and a probable side product of the synthesis, 5-ethoxy-1(3)-(2-morpholinoethyl)-2-[2-(morpholino)ethylthio]benzimidazole (**X**), in addition to probable hydrolysis and oxidation products of **I** (**II**, **III**, **IV**, **V**) (Fig. 2).

However, it was shown during the studies that all standards (synthetic intermediates and probable hydrolysis and oxidation products of **I**) were not completely separated using MeOH:phosphate buffer (0.02 M, pH 7.3) (1:1). Changing the ratio of organic and aqueous phases and the pH of the aqueous phase did not produce better results.

All standards and **I** were completely separated after adding acetonitrile to the mobile phase (column 250 mm × 4.6 mm, $C_{18}$ sorbent, 5 μm) [Luna $C_{18}$(2)]. The best results were observed for mobile phase $CH_3CN$:MeOH:phosphate buffer (0.02 M, pH 7.3) (100:100:240, v/v/v). UV spectrophotometry confirmed that the position of the maximum for **I** in this phase was practically unchanged at the wavelength proposed for detection, 302 nm.

Figure 3 shows a chromatogram of a model mixture of **I** and standards at a content about 0.5% of that of **I**.

Table 1 presents retention times, detection limits at 302 nm, and separation parameters of **I** and the standards.

Based on the results, the method for determining the impurities was modified as follows. Chromatography was carried out over a $C_{18}$ column (250 × 4.6 mm, 5 μm) or an analogous one using a mobile phase of $CH_3CN$:MeOH:phosphate buffer (0.02 M, pH 7.3) (100:100:240) at flow rate 1 mL/min. Isocratic elution was used. The column was at room temperature. The detection wavelength was 302 nm. The concentration of the test solution was 1 mg/mL. The sample volume was 20 μL.

The parenteral solution of **I** (1 mL) was placed into a 10-mL volumetric flask, adjusted to the mark with mobile phase, and stirred. The resulting solution was chromatographed at least five times for each solution.

The content of a single impurity in the drug was estimated by the internal standard method (the standard was a solution of the working standard of **I** in mobile phase at a concentration of 0.005 mg/mL).

The suitability of the chromatographic system was checked by preparing a solution of **I** (0.005 g) and 5-ethoxy-2-mercaptobenzimidazole (0.005 g) in mobile phase (100 mL). The suitability of the chromatographic system was evaluated from the efficiency of the chromatography column (at least 4,000 theoretical plates), the asymmetry coefficient of the peak for **I** (less than 1.5), the separation coefficient of the peaks for **I** and 5-ethoxy-2-mercaptobenzimidazole (at least 6), and the relative standard deviation calculated from the peak areas of **I** in five parallel determinations (less than 2%).

Analysis of serial samples of the parenteral solution of **I** showed that up to five impurities with relative retention times of about 0.27, 0.33, 0.45, 0.55, and 0.72 were present in the samples. The relative retention times of three of the detected impurities agreed with those for a synthetic intermediate of **I** (**IX**, 0.55) in addition to **II** (0.33) and **V** (0.45), two of the probable hydrolysis and oxidation products of **I**. Adding standards to the samples and performing the chromatography under altered conditions (changing the ratio of mobile phase components) showed that the impurities in the solutions and these compounds were most likely identical. The impurities with relative retention times 0.27 and 0.72 could not be identified.

The content of each of the detected impurities as determined by the internal standard method was less than 0.2%. Their total content was less than 0.7%.

**TABLE 4.** Quantitative Determination of Model Solutions of **I** Using HPLC

| Model solution No. | Added (% of reference solution concentration) ($X_i$, %) | Found (% of reference solution concentration) ($Y_i$, %) | Found (% of added) $Z_i = 100 \cdot (Y_i/X_i)$, % |
|---|---|---|---|
| 1 | 118.23 | 119.24 | 100.85 |
| 2 | 113.3 | 113.22 | 99.93 |
| 3 | 108.37 | 108.93 | 100.52 |
| 4 | 103.45 | 103.94 | 100.47 |
| 5 | 98.52 | 98.61 | 100.09 |
| 6 | 93.6 | 93.84 | 100.26 |
| 7 | 88.67 | 89.19 | 100.59 |
| 8 | 83.74 | 83.68 | 99.93 |
| 9 | 78.82 | 78.26 | 99.29 |
| Average $Z$, % | | | 100.21 |
| Standard deviation $S_Z$, % | | | 0.47 |
| Linear regression ($y = bx + a$) parameters | | | |
| a | $S_a$ | $b$ | $S_b$ | $r$ |
| – 1.938 | 4.56 | 1.022 | 0.046 | 0.999 |

**TABLE 5.** Quantitative Determination of Serial Samples of Parenteral Solution of **I** Using HPLC

| Series No. | Quantitative content, mg/mL | Metrological characteristics ($P$ = 95%, $n$ = 5) | | |
|---|---|---|---|---|
| | | S | $\Delta\bar{X}$ | $\bar{\varepsilon}$, % |
| 1 | 10.53 | 0.101 | 0.125 | 1.19 |
| 2 | 9.78 | 0.053 | 0.062 | 0.64 |
| 3 | 10.13 | 0.134 | 0.167 | 1.64 |
| 4 | 9.77 | 0.084 | 0.104 | 1.09 |
| 5 | 10.08 | 0.050 | 0.061 | 0.62 |

Quantitative determination of **I** in the parenteral solutions was carried out by a modified method using UV spectrophotometry and HPLC.

The method for quantitative UV spectrophotometric determination of **I** in the solutions was developed using model solutions. The solvent for the samples was HCl solution (0.01 M). The working wavelength corresponded with the maximum in the UV spectrum of **I**, 302 nm. Measurements were made using a 10-mm cuvette.

The optical density was a linear function of the test solution concentration in the range 0.001 – 0.03 mg/mL of **I**. The correlation coefficient was +0.998. Based on the results, a working concentration of 0.015 mg/mL (optical density about 0.620) was selected.

Quantitative analysis of model solutions of **I** (10 mg/mL ± 20%) and serial samples of the parenteral form (10 mg/mL) was performed under the aforementioned conditions. The parenteral solution of **I** was diluted to the working concentration using HCl solution (0.01 M). A weighed portion of working standard was dissolved in HCl (0.01 M). Table 2 presents the analytical results for the model solutions.

The quantitative content of **I** in the serial samples of the parenteral solution that was determined by UV spectrophotometry was from 9.76 to 10.48 mg/mL. The experimental uncertainty was less than 1.2% (Table 3).

The development of the HPLC method for quantitative determination of the parenteral solution of **I** included a study of the detector response (peak area) as a function of the solution concentration. The function was linear in the concentration range 0.0004 – 0.4 mg/mL (correlation coefficient 0.999) and was validated within the concentration interval from 0.0064 to 0.0096 mg/mL (0.008 mg/mL ± 20%). The correlation coefficient was 0.999. The working concentration was 0.008 mg/mL. Table 4 presents analytical results for model solutions.

Test solutions for quantitative determination and identification of **I** in the parenteral form were prepared as follows. The drug (1 mL) was placed into a 50-mL volumetric flask, adjusted to the mark with mobile phase, and stirred. The resulting solution (1 mL) was placed into a 25-mL volumetric flask, adjusted to the mark with mobile phase, and stirred.

The resulting solutions were chromatographed under the conditions for determining the impurities. The quantitative content of **I** in the parenteral form was determined by the internal standard method by comparing peak areas of the test samples with that of a working standard of **I** (concentration 0.008 mg/mL).

The developed method was used for quantitative determination of **I** in the serial solutions, which gave values from 9.77 to 10.53 mg/mL. The experimental uncertainty was less than 1.7% (Table 5).

It was proposed based on the test results that the authenticity of the parenteral solution of **I** could be determined using UV spectrophotometry and HPLC and the correspondence of the spectral maxima and retention times of the test solution and a standard solution. TLC and HPLC could be used to determine the purity of the parenteral solution of **I**. However, we preferred the HPLC method as a universal one that could determine the authenticity, purity, and quantitative content under identical experimental conditions. The UV spectrophotometric method for determining the quantitative content of the solution of **I** gave more accurate results than the HPLC method. However, the HPLC method was proposed for quantitative determination because of the aforementioned reasons.

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