STRUCTURE OF CHEMICAL COMPOUNDS, METHODS OF ANALYSIS AND PROCESS CONTROL

DEVELOPING ANALYTICAL METHODS FOR THE CREATION OF THE STATE REFERENCE SAMPLE OF NOOPEPT

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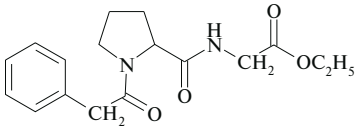
The physicochemical properties of noopept, a new domestic original drug with nootropic action, have been studied with a view to developing analytical methods, establishing quality criteria for the parent drug substance, and creating the state reference sample (SRS). These methods will be included in a draft of the regulation on the SRS of noopept.

Noopept is a new original nootropic drug that was synthesized and studied pharmacologically at the Zakusov Institute of Pharmacology of the Russian Academy of Medical Sciences (ZIP RAMS). The chemical structure of noopept is the ethyl ester of *N*-phenylacetyl-L-prolylglycine [1].



Research on the analysis and standardization of the new drug led to the development of an analytical procedure for noopept in the parent substance and the drug form, tablets [2]. However, further research showed that the development procedures had several drawbacks. Thus, the developed method for quantitative determination of noopept parent substance, a reverse titration method using preliminary hydrolysis, is irreproducible and inaccurate. Furthermore, it was demonstrated that the developed TLC procedure for determining impurities in noopept is not sensitive enough [3]. Therefore, the need arose to develop new, more sensitive, accurate, and reproducible procedures for qualitative and quantitative analysis of the drug. This problem was solved by selecting physicochemical analytical methods. Structural features of noopept enable physicochemical methods based on UV absorption of the drug such as UV spectrophotometry and high-performance liquid chromatography (HPLC) with spectrophotometric detection to be used to analyze its quality.

The main goal of our research was to study the physicochemical properties and develop an analytical procedure of noopept parent substance to create a state reference sample (**I**).

EXPERIMENTAL

Five samples that were synthesized at ZIP RAMS were analyzed. The external appearance and solubility of **I** were studied. The melting point, mass loss on drying, specific rotation of a solution of **I** in $CHCl_3$ (2%), transparency, color, and pH of aqueous solutions (0.5%) of the drug were determined.

UV, IR, PMR, and mass spectra of all samples of **I** were recorded for the study of the spectral characteristics and to confirm its authenticity. UV spectra were recorded on a UV-1700 spectrophotometer (Shimadzu, Japan) using ethanol (95%) as the solvent. IR spectra in KBr disks were recorded on a Perkin—Elmer 457 (Sweden) IR spectrophotometer. PMR spectra in deuterated chloroform ($CDCl_3$) and deuterated dimethylsulfoxide (DMSO-d_6) were recorded on an AC-250 (Bruker, Germany) NMR spectrometer with tetramethylsilane (TMS) internal standard. Mass

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spectra were obtained from the analytical section of FGUP CDC ARRIPC in a SSQ-710 (Finnigan-MAT, USA) mass spectrometer.

An HPLC procedure for analysis of the purity of **I** was developed on an LC-10AT liquid chromatograph (Shimadzu, Japan) with a fixed volume loop (20 μL) and a UV—VIS SPD-10A spectrophotometric detector with variable wavelength.

The stability of samples of the parent substance of **I** was studied during storage by accelerated ageing at 50°C for 46 and 92 d, which corresponded to 1 and 2 yr storage under natural conditions. Furthermore, samples of the parent substance of **I** were stored under natural conditions for 2 yr.

RESULTS AND DISCUSSION

Compound **I** is a white crystalline powder that is poorly soluble in water, readily soluble in ethanol (95%) and $CHCl_3$, and poorly soluble in ether.

The study of the physicochemical properties of aqueous solutions of **I** showed that solutions (0.5%) are transparent and colorless with pH values in the range 6.0 – 6.5. The melting point of **I** fell in the range 95.5 – 97.5°C. The specific rotation determined for a solution (2%) in $CHCl_3$ was from –116.5 to –119.0°. The mass loss on drying was determined at 60°C because the drug has a low melting point. The mass losses were less than 0.5% and were actually from 0.075 to 0.1%.

The UV spectrum of a solution (0.04%) of **I** in ethanol has maxima in the wavelength range 230 – 300 nm at 253 ± 2, 259 ± 2, and 265 ± 2 nm. The peak at 259 nm is the most distinct of these. The specific absorption coefficient at this wavelength was determined for solutions of **I** in ethanol and was 5.96 ± 0.08.

The IR spectrum of **I** in KBr disks had the following characteristic absorption bands (cm^{-1}): 1754 (ester carbonyl, $–COOC_2H_5$), 1695 (peptide carbonyl, –CONH), 1636 (*N*-acyl carbonyl, N–CO–), 1580 – 1600 and 1450 – 1500 (C=C of substituted benzene), 675 – 890 (*p*-substitution of benzene ring).

The PMR spectrum was recorded on an NMR spectrometer at operating frequency 250 MHz at 25°C for solutions of **I** (2%) in DMSO-d_6.

The PMR spectrum of a solution of **I** (2%) taken at 25°C showed a double set of proton resonances for CH_2Gly, NHGly, and CαPro, which is a consequence of the different (*trans*/*cis*) orientation of substituents relative to the tertiary amide bond –C(O)–N<, like for other proline derivatives [4]. The chemical shifts of the NHGly proton as a function of solvent polarity ($CDCl_3$, DMSO-d_6), temperature, and substance concentration showed that the NHGly proton of the *trans*-conformer forms an intramolecular H-bond (IMHB) with the oxygen of the *N*-phenylacetyl, as reported previously [4, 5]. The chemical shift of the NHGly proton of the *trans*-conformer is practically independent of the solvent, which confirms that it forms an IMHB. The resonance of this proton typically has a smaller $\Delta\delta$ ($\delta_{DMSO-d_6} - \delta_{CDCl_3}$). Thus, $\Delta\delta$ for the *trans*-conformer is 0.81 ppm whereas that for the *cis*-conformer is 2.07 ppm.

PMR spectrum of **I** (DMSO-d_6, δ, ppm): 1.17 (3H, t, CH_3CH_2O–), 1.70 – 2.25 (4H, m, 3-CH_2, 4-CH_2Pro), 3.35 – 3.50 (2H, m, 5-CH_2Pro), 3.66 (2H, s, $CH_2C_6H_5$), 3.79 (2H, d, CH_2Gly), 4.08 (2H, q, CH_3CH_2O–), 4.31 (1H, dd, 2HPro), 7.14 – 7.35 (5H, m, $CH_2D_6H_5$), 8.29 (1H, t, NHGly) (*trans*-conformer); 1.16 (3H, t, CH_3CH_2O–), 3.62 (2H, s, $CH_2C_6H_5$), 3.85 (2H, d, CH_2Gly), 4.10 (2H, q, CH_3CH_2O–), 4.17 (1H, dd, 2HPro), 8.63 (1H, t, NHGly) (*cis*-conformer).

The ratio of integrated intensities of resonances for the glycine NH protons in the *trans*- and *cis*-conformers was estimated as 40:60.

Chemical shifts in all studied samples of **I** were identical.

Mass spectra of samples of **I** were obtained at ionizing-electron energy 70 eV, ionization chamber temperature 150°C, sample temperature up to 350°C, heating rate 2.7°C. The mass spectrum of **I** exhibited a peak for the molecular ion with *m*/*z* 318 that corresponded with the molecular structure of noopept. The fragmentation of the molecular ion also agreed completely with the structure of the ethyl ester of *N*-phenylacetyl-*L*-prolylglycine. The spectrum contained peaks due to cleavage from the molecular ion of OC_2H_5, $NHCH_2COOC_2H_5$, $CONHCH_2COOC_2H_5$, and the ions $C_6H_5CH_2^+$ and $C_4H_8N^+$.

Mass spectrum (*m*/*z*, I_{rel}, %): 318 (27), 273 (7), 216 (8), 188 (65), 118 (5), 91 (60), 70 (100).

The ratio of intensities of characteristic peaks in the spectrum remained constant during the whole vaporization process and indicated that the drug did not undergo thermal destruction under the conditions of the mass-spectrometric analysis.

These investigations enabled the UV, IR, PMR, and mass spectroscopic methods to be recommended for confirming the authenticity of **I**.

Impurities in **I** could be starting materials and intermediates from the synthesis of the parent substance. These include phenylacetic acid (PAA), *N*-phenylacetyl-*L*-proline (PAP), and the hydrolysis product of **I**, *N*-phenylacetyl-*L*-prolylglycine (PAPG).

The thin-layer chromatographic (TLC) procedure on Kieselgel 60 F_{254} plates (Merck) for analyzing noopept that was developed earlier enabled individual impurities of at least 1% content to be observed [3]. Further research on the improvement of the TLC procedure for noopept analysis did not significantly increase the sensitivity. Therefore, an HPLC procedure was developed to estimate the impurity content in noopept.

The absorption spectra of solutions of **I**, PAP, PAA, and PAPG in alcohol in the range 200 – 350 nm were studied in order to select the analytical wavelength for the spectrophotometric detector. Judging from the absorption of solutions of **I**, solutions with concentrations of 0.4 mg/mL were prepared

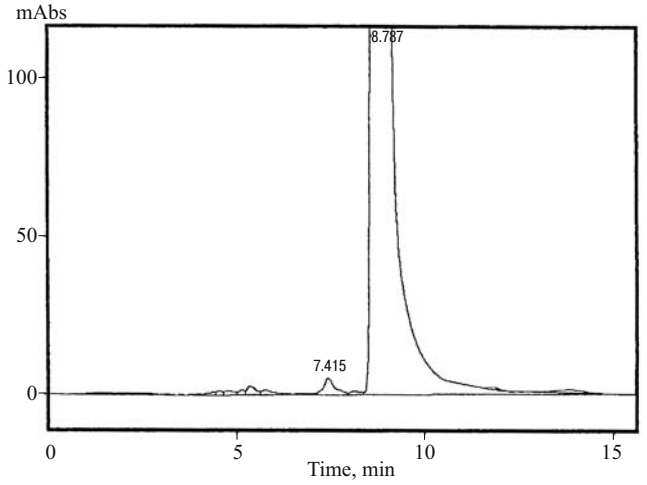

Fig. 1. Typical chromatogram of a standard samples of noopept parent substance.

to record absorption spectra in the range 220 – 350 nm. For absorption spectra in the range 200 – 250 nm, the concentration was 0.004 mg/mL. It was shown that the spectrum of **I** had the following maxima (nm): 205 ± 2 (ε = 1920), 253 ± 2 (ε = 170), 259 ± 2 (ε = 200), and 265 ± 2 (ε = 150). The spectra of PAP, PAA, and PAPG had the same maxima as that of **I**. The absorption in the spectra of **I** and its impurities was much greater at 205 nm than at 259 nm. In order to ensure that the procedure was highly sensitive, the wavelength 205 nm was chosen as the analytical one for determining the impurity content.

The following conditions were selected based on the chromatographic mobility of **I**: Ultra 5 μLm C_{18} column, 250 mm × 4.6 mm with an Ultra 5 μLm C_{18} precolumn, 30 mm × 4.6 mm. Mobile phase: CH_3CN:H_2O:CH_3COOH (glacial) (500:500:1), pH 3.6 ± 0.2, isocratic elution, eluent flow rate 0.5 mL/min, room temperature, sample volume 20 μLL, sample dissolved in mobile phase.

The maxima in spectra of **I** and its possible impurities in the solvent mixture CH_3CN:H_2O (purified):CH_3COOH (glacial) (500:500:1) in the range 200 – 350 nm coincided with those of alcohol solutions. The relative retention times under the selected chromatographic analysis conditions for PAP, PAPG, **I**, and PAA were 0.84, 0.64, 1.00, and 1.08, respectively.

Solutions of **I** in mobile phase (0.4 mg/mL) were prepared in order to observe the impurities in samples of the parent substance of **I**. Figure 1 shows a chromatogram of the noopept solution.

The quantitaties of impurities were estimated using calibration coefficients. Calibration coefficients had to be used to calculate the impurity contents because the concentration (0.4 mg/mL) under the analytical conditions was outside the limits of the linear response range of the detector for solutions of **I**. If lower concentrations of solutions of **I** were used, impurities with a content less than 1% would not be observed on the chromatogram.

The calibration coefficients were determined by preparing model mixtures of **I**, PAPG, PAP, and PAA in various amounts at concentrations 0.0040, 0.0032, 0.0024, 0.0016, 0.0008, and 0.0004 mg/mL.

Calibration coefficients were calculated using the formula:

$$K_i = \frac{S_{ST} C_x}{S_x S_{ST}},$$

where C_x is the concentration of the determined substance, mg/mL; C_{ST} is the concentration of **I**, mg/mL; and S_x and S_{ST} are the peak areas of the determined substance and **I** in integrator units.

The ratio of the concentration and peak area of **I** was taken as 1 so that the calibration coefficient of **I** was 1.

The investigations resulted in the following coefficients: PAPG, 1.04; PAP, 0.99; PAA, 1.21.

Then, the ratio of peak areas of **I** upon injection into the chromatograph of solutions with concentrations 0.4 and 0.004 mg/mL was studied. A calibration coefficient for the peak of **I** in the chromatogram of the solution with a concentration of 0.004 mg/mL was calculated relative to the peak area of **I** in the chromatogram of the solution with a concentration of 0.4 mg/mL.

The calculations were made using the formula:

$$K = \frac{S_{conc.} S_{dil.}}{S_{dil.} C_{conc.}},$$

where $S_{conc.}$ and $C_{conc.}$ are the area of the scaled peak and the concentration of the solution of **I** for 0.4 mg/mL and $S_{dil.}$ and $C_{dil.}$ are the peak area and concentration of the solution of **I** for 0.004 mg/mL.

The coefficient calculated from the formula was 0.4054.

The correction coefficients for PAPG, PAP, and PAA were recalculated taking into account the resulting coefficient and were 0.420, 0.400, and 0.491, respectively.

The contents of each pure impurity in percent (X_i) were calculated using the formula:

$$X_i = \frac{100 S_i K_i}{\Sigma S},$$

where S_i is the peak area of an individual impurity, K_i is the recalculation coefficient for each individual impurity, and ΣS is the sum of peak areas for all impurities taking into account the recalculation coefficient for each individual impurity and the peak area of **I**.

The suitability of the chromatographic system was checked by preparing solutions of **I** and PAA at concentrations of 0.004 mg/mL. The suitability of the system was checked by determining the separation coefficient of the peaks in the mixture, the asymmetry factor of the peak for **I**,

and the relative standard deviation of separate measurements. The efficiency of the chromatographic column was evaluated from the number of theoretical plates calculated for the peak of **I**. The experimental separation coefficient of **I** and PAA should be less than 1.5; the relative standard deviation, less than 2%; the number of theoretical plates, at least 6,000.

Using the developed procedure, the impurity content in the parent substance of **I** was determined. An impurity of PAP, the content of which was less than 0.3% and actually from 0.04 to 0.25% was observed in all samples of **I**. Other impurities were not observed in **I**.

The stability of **I** on storage was studied by the accelerated ageing method and under natural conditions for 2 yr. Samples were analyzed before the test and after the storage time expired for the following quality indicators: external appearance, transparenty, color, pH of a solution, melting point, specific absorption coefficient, and impurities. It was established that the quality of the samples during storage did not change. Investigations of the stability of **I** during storage are continuing.

The developed analytical procedures will form the basis of a draft regulation for a state reference sample of noopept.

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