Noopept: Development and Validation of a UV-Vis Spectrophotometric method for the quantification of (*S*)-*N*-phenylacetyl-L-prolylglycine ethyl ester in bulk drug substance

Lyudmyla Antypenko*[1], Bogdan Burlaka[2], Igor Belenichev[3]

[1]Organic and Bioorganic Chemistry Department, Zaporizhzhya State Medical University, Zaporizhzhya, Ukraine
[2]Technology of Drugs Department, Zaporizhzhya State Medical University, Zaporizhzhya, Ukraine
[3]Pharmacology and Medical Recipes Department, Zaporizhzhya State Medical University, Zaporizhzhya, Ukraine

Summary

A simple UV-spectrophotometric method has been developed and validated for the quantification of noopept in bulk substance. The linearity with r^2 = 0.999 was found at the 258 nm in the range of 0.6 to 1.8 mg/ml in both 5% glycerin-water (w:v) solutions with prior dilution of the analyte in 96% ethanol and after heating at 80-100ºC in a water bath. The limits of detection for above-mentioned cases were found to be 0.14-0.16 mg/ml, while the limits of quantification were 0.42-0.49 mg/ml. The methods were validated for linearity, accuracy, precision, range, ruggedness and robustness.

KEY WORDS: Noopept, (S)-*N*-phenylacetyl-L-prolylglycine ethyl ester validation, UV-spectrophotometry

1. Introduction

Noopept, ((*S*)-*N*-phenylacetyl-L-prolylglycine ethyl ester) is a peptide drug with nootropic and neuroprotective activity (**Figure 1**)[1,2].

It was chosen among other *N*-acylprolyl-containing dipeptides for consequent investigations as the most potent to prevent memory decline evoked by maximal electroshock in a passive avoidance step-through paradigm[3,4]. The substance activity was based on its antioxidant and anti-inflammatory effects, its inhibitory activity towards the neurotoxicity of excess of calcium and glutamate, and its ability to improve blood rheology[5,6]. It was demonstrated, that noopept affected synaptic transmission in central neurons[7], decreased activity of stress-induced kinases, and increased expression of neutrophines in rat hippocampus[8]. Activation of NMDA receptors was involved in the effects of a single injection of the noopept, whereas activation of quisqualate/AMPA receptors was associated with the decrease in their efficacy after repeated use of drug[9]. Besides it was reported, that it improved spatial memory and increased immunoreactivity to Aβ amyloid in an

Corresponding author: Dr. Lyudmyla Antypenko, E-mail: antypenkol@gmail.com Tel.: +380669658740

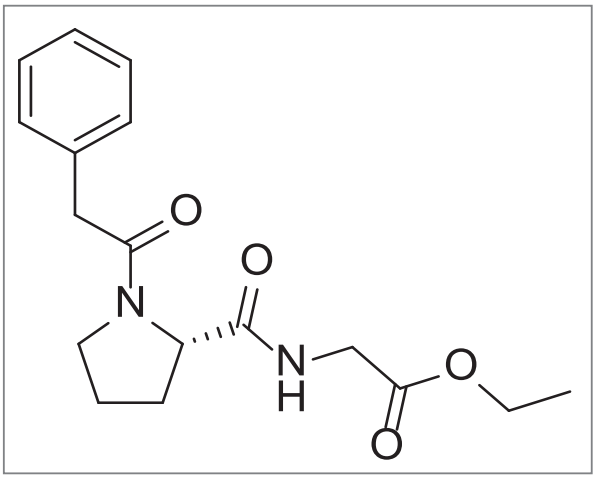

Figure 1: *Noopept structural formula*

Alzheimer's disease mice model following olfactory bulbectomy operation[10]; displayed anxiolytic effects and was used in anxiety treatment[4,7,11].

Furthermore, it was found, that due to hydrophobic interactions with toxic amyloid oligomers of α-synuclein, it prompted their rapid sequestration into larger fibrillar amyloid aggregates, reducing the cytotoxic effect on neuroblastoma SH-SY5Y cells, thus preventing neurodegenerative disorder characterized by α-synuclein containing Lewy body formation and selective loss of dopaminergic neurons in the substantia nigra (Parkinson's disease)[12]. Moreover, its antioxidant and anti-inflammatory effects have been examined in the context of an antidiabetic agent[13].

Clinical pharmacological study established, that noopept is an effective drug for the treatment of psychoorganic disorders with cerebroasthenic and cognitive disturbances in doses of 15 and 30 mg per day[14]. It was found, that in 0.1 and 0.5 mg/kg it effectively eliminated the manifestations of learned helplessness neurosis after long-term treatment[15]. Additionally it was in 200-50,000 times more potent than piracetam as a nootropic agent[16], and produced positive effect in animal models in 0.01 to 0.8 mg/kg dosage[9,10,17].

Considering noopept analytical determination, some instrumental procedures were found in literature. Thus, it was reported, that UV spectra of its 1.0 mg/mL solutions in 95% ethanol had absorption maxima at the 253±2, 258±2 (the strongest), and 265±2 nm[18, 19]. Complete separation of the peaks for extracted excipients, noopept, and its impurities has been achieved by HPLC using mobile phase consisting of acetonitrile, water and glacial acetic acid (500:500:1, v:v) or acetonitrile and phosphate buffer (0.02 M, pH 2.7) (300:800, v:v). The UV absorbance at the 205 nm was selected as the analytical wavelength for determining the content of impurities and 258 nm - to evaluate the dose uniformity, dissolution, and the quantitative determination of active substance. Besides, it was found, that noopept, administered to experimental animals, was subjected to ester hydrolysis, amide hydrolysis, *N*-deacylation and aromatic hydroxylation forming *N*-phenylacetyl-L-prolylglycine, *N*-phenylacetyl-L-proline and L-prolylglycine[20]. Boiko et al. also performed quantitative determination of noopept in the blood plasma using HPLC with UV detection, but at the 206 nm in 0.1 M methanol - ammonium monosubstituted phosphate buffer at pH = 4.6 (40:60, v:v)[21]. Moreover, it was shown, that noopept has been determined by mass spectrometric detection[20,22] or gas chromatography with flame-ionization[20].

In addition, it's worth to mention, that nootropics are under consideration by forensic toxicology, and noopept is recently identified in illegal designer herbal products distributed in Japan, extracted with methanol under ultrasonication along with other substances: an opiate-like analgesic MT-45 (1-cyclohexyl-4-(1,2-diphenylethyl)piperazine, synonym: I-C6), noopept (GVS-111), and two synthetic cannabinoids: A-834735 and QUPIC N-(5-fluoropentyl) analog (5-fluoro-PB-22)[23,24]. The substances were analyzed in high details by: ultra-performance liquid chromatography-electrospray ionization-mass spectrometry (UPLC-ESI-MS), gas chromatography–mass spectrometry (GC-MS) in the electron ionization (EI), liquid chromatography-quadrupole time-of-flight mass spectrometry (LC–QTOF–MS) with a photodiode array (PDA) detector, digital polarimeter. The structural assignments were made *via* ^{1}H, ^{13}C NMR, heteronuclear multiple quantum coherence (HMQC), heteronuclear multiple-bond correlation (HMBC), double quantum filtered correlation spec-

troscopy (DQF-COSY), and rotating-frame nuclear Overhauser effect (ROE) spectra. The UV analytical wavelength was also reported to be 258 nm.

Summing up, although the noopept structure and pharmacological profile has been fully described, no simple validated, detailed UV-method has been reported to determine noopept in bulk drug substance. Thus, our aim was to develop, validate and propose such a procedure in this paper.

2. Materials and methods

2.1. Instrumentation

The substance was weighed using analytical balances Shimadzu AUX220 (10 mg - 220 g), Shimadzu Corporation, ShimUkraine Ltd., Kyiv. UV spectra were recorded on a UV-*vis* spectrophotometer UV-2600 (190-1100 nm), Shimadzu Corporation, ShimUkraine Ltd., Kyiv.

2.2. Reagents and solutions

The used ethanol was of 96% (v:v) concentration and medical purity: "Pharmasept", Lohvytsky alcohol factory, Chervonozavodske, Ukraine. Glycerin was of 99.5% concentration and medical purity (GOST 6824-96): SynbiaS, Kiev, Ukraine. Working substance of noopept (CAS Number 157115-85-0) was purchased from Shijiazhuang Prosperity Import and Export Co., Ltd., China. Manufacture Date 2016/10/01. Purity: ≥98%. Distilled water was used throughout all experiments.

2.3. Validation

Calibration curve. The initial standard solution (0.1%) was prepared by the following procedure: a) dissolve 0.0500g of noopept in a 50 ml volumetric flask with 1 ml of 96% ethanol, stir for 5 min, then fill with 5% glycerine-water (w:v) solution, stir for 5 min, and left for more 15 min; b) dissolve 0.0500g of noopept in a 50 ml flask containing 5% glycerine-water (w:v) solution, place the solution in a water bath for 5 min at 80-100ºC., stir for 5 min, and left to cool down for 20 min. Quality control solutions were prepared over the concentration range 0.2 to1.8 mg/ml (0.02-0.18%) in the same manner. All solutions were stored at the 18-22°C.

The calibration curve was constructed by measuring absorption at λ_{max}, 5 times for each sample. *The regression equation* was obtained by least squares regression analysis for n=5. Regression equation: Y = slope × C + intercept. Slope, intercept and regression coefficient were determined from the regression analysis calculations in Microsoft Excel 2007[25].

Using this linear equation, *regression coefficient* (r^2) and the *detection limits* were calculated. *Accuracy*: mean ± SD; *Linearity* (lowest – highest concentration while curve is linear); *SE of intercept*: √ of $\sum(y-y')/n)$, where y - standard concentration, y' - found concentration; *SD of intercept*: SE of intercept×√n.

The limit of detection (LOD): 3.3×(SD of intercept / slope); and the *limit of quantitation* (LOQ): 10×(SD of intercept / slope).

Precision (repeatability of the method) was evaluated by repeated absorption measurements at λ_{max} and the results were expressed as the mean standard deviation (SD) and the percent relative standard deviation RSD (%). To evaluate intra-day and inter-day precision the samples were analyzed six times a day and for 6 consecutive days.

3. Results and discussion

According to the safety sheet data of Caiman Chemical Company the solubility of Noopept (CAS Number is 157115-85-0) is: ~1 mg/ml in PBS, ~ 20 mg/ml in DMSO, ~ 25 mg/ml in DMF [26], freely soluble in chloroform, 95% ethanol, slightly soluble in water, very slightly soluble in diethyl ether[19].

In a paper regarding HPLC analysis of noopept it has been reported that by changing the ratio of mobile-phase components towards an increased aqueous content the selectivity of the procedure was increased; however, the efficiency was reduced due to hydrolysis of noopept[18]. After one day in an aqueous medium, slow hydrolysis of the ester bond occurred and within a month 1% of the free acid was formed; in 0.1M HCl solution - the next day 15% of impurities have been detected; in 0.1M NaOH solution - the next day 99% of noopept was hydrolyzed[19]. Hydrolysis of the amide bond during a month was not greater than 5%.

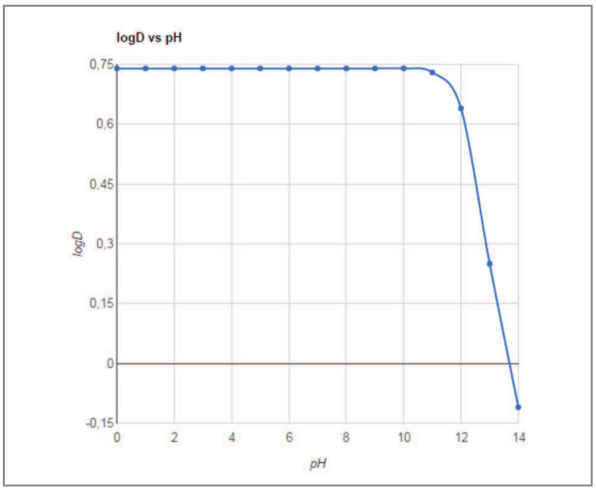

Figure 2: *Noopept logD prediction*

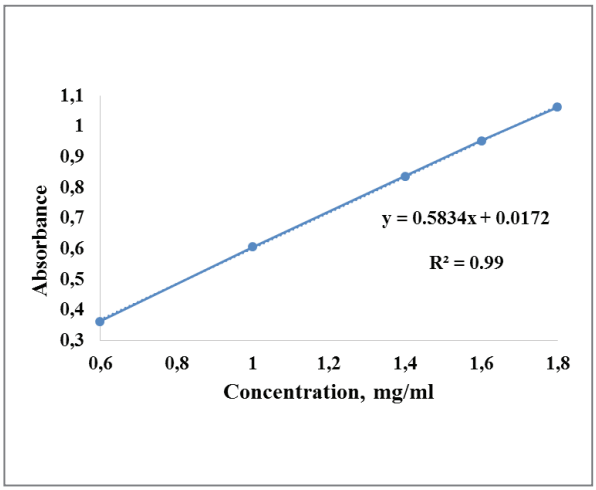

Figure 3: *Calibration curve of noopept in 5% glycerin-water (w:v) solution with prior dilution in 96% ethanol*

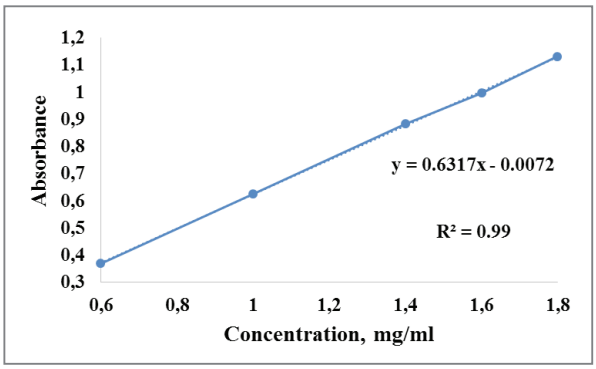

Figure 4: *Calibration curve of noopept in 5% glycerin-water (w:v) solution with heating at the 80-100ºC*

In the light of the above considerations it was decided to use 5% glycerin-water solution as solvent, because of future dialysis studies. Considering noopept bad solubility in this mixture, it was decided firstly to dilute substance in minimum quantity of 96% ethanol, which was found to be 1 ml for 50 ml of main solvent.

The measured pH of the 0.1-0.16% (w:v) working solutions was 5.65-5.76, and close to data of intestines environment. At this pH, noopept should be in non-ionized form according to *in silico* calculations done by LogD Predictor (**Figure 2**)[27].

The absorbance maxima were found as it was reported earlier: 253, 258 and 264 nm in both cases[19].

Validation of the method was prepared in accordance to the analytical method validation parameters[28]. According to the Beer's law, regression coefficient, obtained similar specific absorbance (6), the calibration curve of noopept exhibited good linearity over the concentration range 0.6 to 1.8 mg/ml (**Table 1, Figure 3**).

The above-mentioned data were evaluated by linear regression analysis, which was calculated by the least-square regression analysis measuring maximum absorbance of noopept standard solutions. The concentration range was very close to the data reported earlier for the determination of noopept in 95% ethanol (0.2-1.6 mg/ml)[19], but due to bad recovery because of unequal distribution of noopept in solvent, 0.2 and 0.4 mg/ml were extracted from the linearity calculations.

The precision of the method was evaluated by measuring absorption at λmax of noopept standard solutions for five times. Standard deviations of each measured absorbance were good - 0.0132-0.0225 mg/ml and RSD was 0.01-0.1%.

The recovery at five different concentrations (99.9±0.85%) evaluated the accuracy of the method (**Table 1**). The mean percentage of recoveries was 99.9±0.01%.

The presented calibration curve can be used for fast concentration evaluation of unknown solution of noopept (**Figure 3**).

Table 1. Concentration of standard solutions of noopept in 5% glycerin-water (w:v) solution with prior dilution in 96% ethanol, their absorbancies, accuracy and recovery data

Concentration	A	SD	%RSD	E%cm	[a]Found C.	[b]Recovery
mg/ml	of 5 meas.	mg/ml			mg/ml	%
0.6	0.3626	0.0132	0.03	6.0	0.59	98.7
1	0.6058	0.0187	0.03	6.1	1.01	100.9
1.4	0.8371	0.0144	0.01	6.0	1.40	100.4
1.6	0.9521	0.0225	0.02	6.0	1.60	100.2
1.8	1.0625	0.0189	0.02	5.9	1.79	99.5
					%RSD	0.01

[a]Found concentration: (Absorbance – intercept) / slope;
[b]Recovery: found concentration / labeled concentration×100.

Table 2. Concentration of standard noopept solutions in 5% glycerin-water (w:v) solution with heating at the 80-100° C, their absorbancies, accuracy and recovery data

Concentration	A	SD	% RSD	E% cm	[a]Found C.	[b]Recovery
mg/ml	of 5 meas.	mg/ml			mg/ml	%
0.6	0.3705	0.0026	0.01	6.2	0.60	99.6
1	0.6255	0.0122	0.02	6.3	1.00	100.2
1.4	0.8825	0.0022	0.002	6.3	1.41	100.6
1.6	0.9964	0.0031	0.002	6.2	1.59	99.3
1.8	1.1318	0.0039	0.002	6.3	1.80	100.2
					SD	0.51
					%RSD	0.01
					Accuracy	99.98±0.51
					Recovery	99.98±0.01

[a]Found concentration: (Absorbance – intercept) / slope;
[b]Recovery: found concentration / labeled concentration*100

Moreover, to exclude unnatural reagent for human plasma, namely, - ethanol from the future pharmacological investigations of pharmaceutical dosage forms, it was decided just to heat the glycerin-water solution with noopept in the water bath and compare the obtained results with previous ones (**Table 2, Figure 4**).

Upon heating the noopept solution, according to the increasing absorbance values, a solvatochromic effect was observed, most likely due to noopept protonation and to the formation of stable aggregates in this solvent[29].

Slope, intercept and correlation coefficient were determined from the regression analysis calculations (**Table 3**).

The limits of detection (LOD) were found to be 0.14-0.16 mg/ml upon heating or upon addition of

Table 3. Linearity data, LOD and LQD of noopept in 5% glycerin-water (w:v) solution

Parameters	With addition of 0.5% of 96% ethanol (v:v)	Under heating	Reported data in 95% ethanol[19]
Slope	0.5834	0.6317	0.5921
Intercept	0.0172	0.0072	0.0028
Linearity (mg/ml)	0.6-1.8	0.6-1.8	0.2-1.6 (UV) 0.0025-1.0 (HPLC)
Regression equation	y = 0.5834x+0.0172	y = 0.6317x-0.0072	y = 0.5921x+0.0028
r^2	0.999	0.999	0.999
SE of intercept (mg/ml)	0.0130	0.0118	-
SD of intercept (mg/ml)	0.029	0.026	-
LOD (mg/ml)	0.16	0.14	-
LOQ (mg/ml)	0.49	0.42	-

ethanol respectively, while the corresponding limits of quantification (LOQ) were 0.42-0.49 mg/ml respectively.

The ruggedness of the methods was determined by performing the same assay by different analysts, also while the precision was investigated. In addition, the assay was performed for 0.1% noopept solution during week to check its reproducibility (**Table 4**).

The results were found to be highly reproducible during the day for dilution in the first method - RSD was 0.006%, but for the period of the week it increased to 0.02%, due to ester bond hydrolysis, but still with high valid values. The dissolution in 5% glycerin-water solution influenced practically in the same rate - RSD was 0.01%.

To determine the robustness of the method, experimental conditions like room temperature, stirring time, addition of different ethanol volumes were studied. It is very important to dilute noopept firstly in the 96% ethanol, because without it substance formed suspension in 5% glycerin-water (w:v) solution. It was found, that 1 ml was enough in case of dilution 0.0100-0.0900 g of noopept in a 50 ml volumetric flask. Also heating for 5 min at 80 to 100° C in a water bath was essential to dilute the same quantity of the bulk drug substance. During the week, opacity of solutions has not appeared in both cases. Furthermore, stirring for 5-10 min over a time period 0.5 - 2 h. was essential to determine most accurate data as it was already mentioned in **Table** 4. The temperature has also affected the results, and the 20-22°C was chosen as the most appropriate one for prior dilution in the ethanol and 80-100° C – for the second method with heating, to be calculated by the obtained equations, because otherwise, due to solvatochromism phenomenon, absorbances were of higher values.

Hence, to obtain absorption in the range of 0.6-0.8 and to detect the amount of noopept in bulk by first dilution in ethanol, the next method is proposed.

Quantitatively place 0.0500-0.0700 g of noopept in a 50 ml volumetric flask, dissolve with 1 ml of 95% ethanol, stir for 5 min., add 5% glycerin-water (w:v) solution, stir for 5 min. and left for 30 min. Determine the concentration of noopept by measuring maximum UV absorption at the wavelength of 258 nm in comparison to 5% glycerin water (w:v) solution with addition of 0.5% of 96% ethanol (v/v) (0.1 ml of 96% ethanol with 4.9 ml of 5% glycerin water (w:v) solution) in 3 ml cuvette with 1 cm layer.

The sample concentration is calculated in accordance with the following equation:

$$\text{C mg/ml of final solution} = \frac{Ai - 0.0172}{0.5834}$$

Table 4. The intra-day and inter-day precision of noopept in 5% glycerin-water (w:v) solutions

	With addition of 0.5% of 96% ethanol (v:v)		With heating	
	Intra-day	Inter-day	Intra-day	Inter-day
1	0.5941	0.6051	0.6402	0.6446
2	0.6011	0.5808	0.6398	0.6403
3	0.6001	0.5770	0.6365	0.6416
4	0.5939	0.5702	0.6343	0.6502
5	0.5917	0.5654	0.6315	0.6612
Mean	0.5962	0.5876	0.6365	0.6476
SD	0.0042	0.0152	0.0037	0.0085
%RSD	0.006	0.021	0.005	0.01

$$\text{C \% (w/v) of final solution} = \frac{Ai \cdot 0.1}{0.6004}$$

or concentration in the initial sample:

$$\text{C \% (w/v) in bulk} = \frac{Ai \cdot 0.1 \cdot 50}{0.6004 \cdot l \cdot a}$$

where A*i* – sample absorbance;

0.1 - concentration (%) of the standard solution with absorbance of 0.6004;

50 – flask volume, ml;

l – cuvette layer is 1 cm;

a – sample weight, g.

Furthermore, to determine the amount of noopept with heating, the next method is given.

Quantitatively place 0.0500-0.0700 - for ukrainian rules of accurate weigh of noopept in a 50 ml volumetric flask, dissolve in 5% glycerin-water (w:v) solution by heating in the water bath at the 80-100ºC during 5-10 min. Cool the solution to 20-22ºC for 30-45 min. Determine the concentration of noopept by measuring maximum UV absorption at the wavelength of 258 nm in comparison to 5% glycerin water (w:v) solution in 3 ml cuvette with 1 cm layer.

The sample concentration is calculated in accordance with the next equation:

$$\text{C mg/ml of final solution} = \frac{Ai + 0.0072}{0.6317}$$

$$\text{C \% (w/v) of final solution} = \frac{Ai * 0.1}{0.6245}$$

or concentration in the initial sample:

$$\text{C \% (w/v) in bulk} = \frac{Ai \cdot 0.1 \cdot 50}{0.6245 \cdot l \cdot a}$$

where A*i* – sample absorbance;

0.1 - concentration (%) of the standard solution with absorbance of 0.6245;

50 - flask volume, ml;

l - cuvette layer is 1 cm;

a - sample weight, g.

4. Conclusions

It was found, that noopept in 5% glycerin-water (w:v) solution could be accurately qualitatively and quantitatively determined by UV spectroscopy by measuring the maximum absorbance at the 258 nm with initial addition of 0.5% of 96% ethanol (v:v) or upon heating for 5 min in a water bath at 80-100°C. The method was validated and calibration curves gave good linearity (r^2 = 0.999) over the concentration range of 0.6 to 1.8 mg/ml. The LOD and LQQ were calculated and evaluation criteria such as accuracy, precision, robustness and ruggedness also showed high validity. □

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