Amino Acid Composition of Nephrophyt, a New Complex Plant Preparation and Its Possible Role in Correction of Mercuric Chloride-Induced Acute Renal Failure in Rats

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Amino acid composition of a new Russian nephroprotective drug Nephrophyt (a complex mixture of dry plant extracts) was studied for the first time in order to validate its pharmacological effects in rats with mercuric chloride-induced nephropathy. The concentration of pyrrolidone amino acids and serine in this preparation was extremely high, which is uncommon for plant raw material. These data are in good correlation with pronounced nephroprotective effect and agree with published reports on pharmacological effects of individual amino acids and their metabolites. The prospects of the use of this preparation based on the data on its amino acid composition are discussed.

Key Words: *mercuric chloride-induced nephropathy; Nephrophyt; amino acid composition; phytocorrection*

Nephrophyt is a bioactive food additive containing granulated dry extracts of *Orthosiphon stamineus*, *Polygonum aviculare*, and *Arctostaphylos uva ursi*. The active principle of this additive is the sum of bioactive substances, including amino acids and phenol compounds [6].

Preclinical studies of pharmacological activity, pharmacotherapeutic efficiency, and safety of Nephrophyt, carried out at Laboratories of Experimental Pharmacology and Drug Safety, Institute of General and Experimental Biology, Siberian Division of Russian Academy of Sciences (1999), demonstrated pronounced diuretic, hypoazotemic, antiinflammatory, antibacterial, regeneratory, spasmolytic, antioxidant, membrane-stabilizing, and antistress effects of this additive in a dose of 50 mg/kg. The combination of these effects underlies high efficiency of a nephroprotective agent in nephropathies of different origin [5].

Toxicological studies of Nephrophyt showed that it belongs to a group of practically harmless substances (LD_{50} for mice 1466.7 mg/kg). Nephrophyt exhibits no local irritating effects, is not cumulated in the body, produces no allergenic, mutagenic, teratogenic, and embryotoxic effects and no negative effect on the postnatal development of the progeny [5].

Detailed investigation of chemical composition of the agent is needed for identification of active components determining its pharmacological effects.

We studied the relationship between amino acid composition of Nephrophyt granules and pharmacological effects of the drug on a model of mercuric chloride-induced acute renal failure in rats.

This study was prompted by published data that pyrrolidine imino acids (proline, hydroxyproline) intensely stimulate the formation of granulation and fibrous tissue during recovery of cells and tissues after acute toxic injury to the kidneys [11]. Increased requirement of wound surfaces and chemically damaged tissue for these compounds is determined by intensive collagen synthesis (protein with high content of proline and hydroxyproline) [12,13].

Our study of amino acid composition of the preparation and its effect on the correction of mercuric

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chloride-induced acute renal failure in rats can help to understand the mechanisms of pharmacological effect of this drug.

MATERIALS AND METHODS

Experimental model of mercuric chloride-induced acute renal failure was created by a single intraperitoneal injection of water solution of mercuric chloride in a dose of 2 mg/kg. Experiments were carried out on 1255 random-bred rats of both sexes (160-220 g). Control rats were injected with an equal volume of distilled water according to the same protocol. Intact controls received no manipulations.

The content of amino acid in granules was measured by high performance liquid chromatography on a GILSON 321 chromatograph with spectrofluorimetric detection.

Weighted tissue sample (1,0000 g) was placed into a 250-ml sealed flask containing 50 ml water. Extraction was carried out for 30 min at 50°C in an ultrasonic bath. The extract was cooled to 18-20°C and filtered through a plicated paper filter. The volume was adjusted to 100 ml with water. A 50-ml portion of the extract was mixed with 10 ml hexane in a separating funnel, and extracted twice in order to remove essential oils. Hexane extracts were discarded. An equal volume of chloroform was added to aqueous extract and complete 3-fold chloroform extraction was carried out for degreasing and partial removal of plant pigments. Then 10 ml purified water extract was mixed with 12 ml 96% ethanol and the water-ethanol solution was left for 10 h at 4°C. The precipitate was removed by centrifugation.

Water-ethanol solution was evaporated to a volume of 3-4 ml and 10 ml methanol was added for precipitating polymeric compounds, after which the mixture was filtered through a pore-40 glass filter. The precipitate on the filter was several times washed with methanol and the filtrate was completely evaporated.

In order to detect free amino acids, 100 μl 20 mM HCl was added to the dry sample, mixed, and centrifuged. Borate buffer (60 μl, 10 mM, pH 7.80) and 20 μl derivatizing reagent forming brightly stained complexes with amino acids [1] were added to 20 μl of the resultant solution.

We used a Waters LTR60 metal column (3.9×150 mm; Associates) filled with DCGA-60R polyion-exchange resin, whose matrix granules contain hydrophobic phenyldomains [14]. The mobile phase was as follows: eluent A (sodium acetate—concentrated phosphoric acid—triethylamine water—EDTA; 19:6.9:2:72:0.1%); eluent B (acetonitrile); eluent C (water). The linear acetonitrile gradient (0-20%) was formed by a 3-chamber Waters GLC 20 mixer with the total volume of 80 ml; elution rate 1 ml/h, 1100 p.s.i. (area/inch^2), and 37°C. All reagents were analytically pure (Serva) [14].

The detection was carried out using a spectrofluorimetric detector for primary amines at λ=250 nm and for second amines at λ=395 nm. Chromatograms were analyzed using standard analytical programs [1].

Amino acid content was calculated using Multichrome for Windows 2000 software by the absolute calibration method in conversion to the volume of the analyzed sample in milligrams. For this, derivatives of amino acid calibration standard containing 100 pmol/μl each amino acid (cysteine — 50 pmol/μl) were added in parallel into the chromatograph. Amino acid derivatives were prepared similarly as the studied sample derivative. Amino acids were identified by the calibration standard.

In order to measure bound amino acids in the initial preparation, its water extract was subjected to acid hydrolysis. To this end, dry residue evaporated from water-ethanol solution was transferred (by portions) in 2 ml of 6N HCl to an ampoule, the air was vacuum pumped, and the ampoule was hermetically sealed. Hydrolysis was carried out in a thermostat for 20 h at 105°C. The ampoule was opened, its contents was quantitatively transferred into a flask, and evaporated

TABLE 1. Free and Bound Amino Acids in Nephrophyt Granules

Amino acid	Nephrophyt, 1 mg	
	pmol	μg amino acid
Free amino acids		
serine	407.1	0.043
glutamic acid	13.09	0.0019
glycine	57.64	0.0043
histidine	173.3	0.027
arginine	12.01	0.0021
tyrosine	44.99	0.0081
alanine	5	0.00044
proline	28.28	0.0037
lysine	3.16	0.00046
phenylalanine	2.527	0.00042
Total	747.4	0.091
Bound amino acids		
hydroxyproline	198.2	0.029
asparaginic acid	43.72	0.0058
glycine	13.37	0.001
serine	7.33	0.0008
phenylalanine	3.40	0.00056
Total	618.3	0.037

completely. The dry residue was dissolved in 100 µl 20 mM HCl, mixed, and centrifuged. Amino acids were detected in 20 µl supernatant by the above described method.

Renal function in mercuric chloride-induced acute renal failure was studied by the most informative methods allowing dynamic evaluation of developing changes. Renal function was evaluated by the following parameters: diuresis (routine tests without and with 2.5% water load [2]), sodium and potassium concentrations in the urine (flame photometry on a Flapho-4 device), depuration function of the kidneys (urea and creatinine content in the serum and urine by universal methods with Bio-La-Test Lachema kits), protein content in the urine was evaluated by the sulfosalycilic acid test [4]; and glomerular filtration rate was evaluated by clearance of endogenous creatinine [2].

The significance of differences between the groups was evaluated by methods of variation statistics. The data were processed using Wilcoxon—Mann—Whitney nonparametric *U* test [3]. The differences were considered significant at $p \leq 0.05$.

RESULTS

Dry extract is characterized by extremely high serine content in the pool of free amino acids (Table 1), while the content of this oxyamino acid in the preparation proteins is not high. The content of bound serine is 58 times below its content in the fraction of low-molecular weight substances. This is a unique feature of this plant preparation: the ratio of free and bound amino acids in raw plants usually did not exceed 6-8 [10]. Moreover, it was shown [8,15] that addition of serine and threonine to polyvitamin mixtures potentiates their normalizing effects on the cation-filtering function of kidneys in rabbits with ergotamine vasoconstrictor ischemia. This fact attracts attention to a small group of oxyamino acids as components of plant extracts whose potentialities in the correction of renal dysfunctions deserves detailed investigation.

One more characteristic of amino acid composition of Nephrophyt is very high content of hydroxyproline in high-polymeric fraction and high content of its analog and metabolite proline in free amino acids (Table 1). High content of these amino acids is typical of animal proteins (collagen, protocollagen, osteocalcine, *etc.*), but their content in plants usually does not exceed 8-10 pmol/g dry material [9]. On the other hand, amino acids of pyrrolidone series stimulate epithelialization and cicatrization. Many authors recommend collagen hydrolysates and purified proline and hydroxyproline for the treatment of toxic and autoimmune renal disorders [7,8].

TABLE 2. Effects of Nephrophyt on Renal Function and Dynamics of Accumulation of LPO Products in Albino Rats with Mercuric Chloride-Induced Acute Renal Failure

Parameter	Period of observation, group								
	day 1			day 3			day 7		
	intact	control	nephrophyt	intact	control	nephrophyt	intact	control	nephrophyt
Serum creatinine, µmol/liter	72.40±3.51	351.3±4.9	306.40±9.71*	72.40±3.51	349.6±30.5	303.7±9.3*			
Urinary creatinine, mmol/liter	4.3±0.6	0.8±0.2	3.00±0.13*	4.3±0.6	1.10±0.09	2.80±0.14*	4.3±0.6	3.80±0.34	4.30±0.23*
Free creatinine phosphate, µl/min	488±59	6.80±0.69	40.80±2.41*	488±59	8.90±0.93	56.60±4.15	488±59	250.9±26.5	420.4±10.3*
Diuresis, ml/100 g	0.50±0.02	0.20±0.02	0.30±0.05	0.50±0.02	0.20±0.02	0.30±0.03	0.50±0.02	0.40±0.02	0.50±0.03
Urinary K^+, mg/ml	3.50±0.19	0.90±0.14	0.70±0.07	3.50±0.19	1.30±0.19	1.90±0.04*	3.50±0.19	2.80±0.15	3.40±0.09*
Urinary Na^+, mg/ml	1.2±0.1	0.30±0.03	0.40±0.07	1.2±0.1	0.60±0.09	0.90±0.08*	1.2±0.1	1.1±0.1	1.30±0.09*
Urinary protein, g/liter ($\times 10^{-1}$)	0.40±0.02	2.30±0.15	1.00±0.08*	0.30±0.02	2.30±0.35	0.70±0.08	0.30±0.02	0.70±0.05	0.40±0.03

Note. *$p<0.05$ compared to the control.

The unique characteristics of amino acid composition of Nephrophyt detected in our study can be used for purposeful search for drugs correcting the urinary system dysfunctions, including toxic nephropathies. The results of our analysis of amino acid composition of the extract (Table 1) and clinical parameters characterizing renal function (Table 2) are in line with the conclusions on the efficiency of Nephrophyt in correction of renal function due to its unique composition, qualitatively different from the majority of known plant extracts [7,9].

We believe that the results of our study will help chemists and pharmacologists to investigate the characteristic features of Nephrophyt, determined by its unique composition, and, presumably, explaining its high pharmacological efficiency in toxic aftereffects of nephropathies.

The availability of plants used for the creation of this drug and their relatively low prevalence beyond the territory of the former USSR (excepting Orthosiphon, growing in Asia) [5,12] makes these studies perspective.

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