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Quantification of Marker Compounds in Multi-Component Sedative and Anxiolytic Herbal Product Phyto Novo-Sed based on herbal extracts.

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ABSTRACT

Herbal product (Phyto Novo-Sed) is an aqueous-alcoholic (40% ethanol) extract of the mixture of therapeutic herbal raw materials: motherwort grass (*Leonurus cardiaca L., Lamiaceae* family); lemon balm grass (*Melissa officinalis L., Labiatae* family), hawthorn fruits (*Crataegus sanguinea Pall., Rosaceae* family); rose hip fruits (*Rosa cinnamomea L., Rosaceae* family); purple coneflower grass (*Echinacea purpureae (L.) Moench, Asteraceae* family) in w/w ratio 2:2:1:2:1. Phyto Novo-Sed is used as sedative agent. Previously performed analysis of the extracts of each of the raw materials comprising the product allowed identification of marker compounds, which characterize the particular raw material in the mixture extract. The developed method of marker compound quantification includes 2 sequential runs of HPLC on octadeylcasilane resin with different gradient regimens using a mobile phase, which consists of acetonitrile, tetrahydrofuran, phosphate buffer solution and dimethylformamide. The first chromatography run (gradient type 1) is characterized by slow elevation of the content of strong eluting agent acetonitrile. These chromatography conditions are optimized for assay of ascorbic, chlorogenic and caffeic acids and rutin. Second chromatography run (gradient type 2) is characterized by faster achievement of strong eluting conditions. These chromatography conditions are optimized for assay of hyperoside, quercetin, chicory, rosmarinic and 2-hydroxycinnamic acids. The validated parameters show the developed method to be specific and to have correct repeatability. The content of marker compounds was quantified in 6 batches of the phytoproduct produced by different manufacturers. The content of marker compounds (mg/ml) in samples of Phyto Novo-Sed liquid extract produced by different manufacturers falls into the following ranges (in descending order): ascorbic acid (4,7 - 7,8), rosmarinic acid (0,7 - 1,1), chlorogemic acid (0,3 - 0,9), rutin (0,5 - 0,8), chicory acid (0,3 – 0,5), quercetin (0,2 - 0,4), hyperozide (0,1 - 0,3), caffeic acid (0,1 - 0,3), 2-hydroxycinnamic acid (0,06 – 0,14).

Keywords: phytoproduct Phyto Novo-Sed, marker compounds, HPLC, assay

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INTRODUCTION

Phytotherapy of neurotic disorders is attractive due to mild effect, high safety level and possibility of prolonged use while maintaining high efficacy. Phyto Novo-Sed, which has been developed for this application, contains an extract of an original mixture of 5 therapeutic plants, and demonstrates therapeutic effect on different stages of development of neurotic and neurosis-like conditions. Phyto Novo-Sed shows cardiotonic activity, enhances cardiovascular tolerance of elevated physical strain, stabilizes blood pressure level, and is approved for use as sedative agent [1]. Herbal product Phyto Novo-Sed is an aqueous-alcoholic (40% ethanol) extract of the mixture of therapeutic herbal raw materials: motherwort grass (*Leonurus cardiaca L.*, *Lamiaceae* family); lemon balm grass **(***Melissa officinalis L.*, *Labiatae* family), hawthorn fruits *(Crataegus sanguinea Pall., Rosaceae* family*);* rose hip fruits (R*osa cinnamomea L., Rosaceae* family*);* purple coneflower grass (*Echinacea purpureae* (*L.*) *Moench, Asteraceae* family) in w/w ratio 2:2:1:2:1.

The drug efficacy is determined by pharmacological activity of each of its five raw materials. Motherwort grass extracts and tinctures are used as cardiovascular, hypotensive, sedative agents in cases of increased nervous excitability and early stages of arterial hypertension accompanied by sleeplessness, feeling of tension and increased reactivity, as well as in cases of autonomous vascular dystonia and neuroses [2-7].

Extracts of lemon balm grass show anti-inflammatory, bacteriostatic, antiviral and antioxidant properties, and demonstrate significant relaxing and spasmolytic effects [8-11].

Extracts of purple coneflower grass stimulate cellular and humoral immune reactions and show antimicrobial, antiviral and antifungal effects. The extracts are used as antimicrobial, antiviral, anti-inflammatory and immune stimulating agents after antimicrobial, cytostatic therapy and radiotherapy, as well as in cases of mental and physical overstrain [12-15].

Extracts of hawthorn fruits are effective in cases of cardiac failure, functional cardiac disorders, hypertension and arrhythmia. They are also used to improve digestion, circulation and as antispasmodic, cardiotonic, diuretic, hypotensive and anti-atherosclerotic agents [16-21].

Extracts of rose hip fruits show a wide range of effects: choleretic, diuretic, anti-atherosclerotic, antimicrobial, anti-inflammatory, astringent, wound-healing, sedative, blood coagulating, laxative, hematopoietic, as well as improvement of general condition [22-27].

We have previously developed HPLC methods of separation and identification of components of multicomponent herbal product Phyto Novo-Sed [28]. 17 Main biologically active substances were identified.

This study was aimed at development of a quantitative chromatographic assay of biologically active marker compounds of Phyto Novo-Sed components and at determination of their content in the products.

MATERIALS AND METHODS

Objects: Phyto Novo-Sed liquid extract 50 or 100 ml for oral intake produced by: Pharmaceutical Industrial Company PharmVILAR (Moscow, Russia), Microgen (Moscow, Russia), Vatchem Phamacy (Ryazan, Russia).

Reference standards: chlorogenic acid, rutin, caffeic acid, chicoric acid, rosmarinic acid, 2-hydroxycinnamic acid, ascorbic acid, gallic and salicylic acids were from Sigma-Aldrich Chemi Gmbh, (Steinhein, Germany); hyperoside was from Merck (Darmstadt, Germany); quercetin was from Acros Organics (Geel, Belgium).

Reagents and solvents: potassium dihydrophosphate, analytical grade (Avogadro, Moscow, Russia), ortho-phosphoric acid, special purity grade (Reachim, Moscow, Russia), acetonitrile, HPLC grade (Lab-Scan, Gliwice, Poland), dimethylformamide, analytical grade (ECOS-1, Russia), tetrahydrofuran, HPLC grade and absolute ethanol, analytical grade were from Merck (Darmstadt, Germany).

Equipment and chromatographic condition: HPLC system ProStar (Varian, USA) containing pump ProStar 230 and detector ProStar 325. Column Dr. Maisch Luna 100 C18(2), 250 x 4.6 mm, 5 □. Injection volume 20 □l (Reodyne injector). Mobile phase flow rate 1.0 mL/min; room temperature. Detection at wavelengths 285 and 325 nm.

Preparation of the mobile phase aqueous component: *Phosphate buffer solution, pH 3,15 with dimethylformamide:* dissolve 2.72 g of potassium dihydrophosphate in water in a 1000-ml volumetric flask, add 1 ml of concentrated ortho-phosphoric acid, dilute to volume, mix and adjust the solution pH to 3,15. Add 1.5 ml of dimethylformamide. Filter the obtained solution through membrane filter 0.45 mm.

The following chromatographic parameters were calculated for system optimization: capacity index (k'), efficacy – theoretical plate number (N), selectivity (α) and separation ratio (R_s).

EXPERIMENTAL

Standard solution and quantification: Standard samples were prepared using reference standards; gallic and salicylic acids were used as internal standards. Preparation of standards: 20 mg of each reference standard or gallic/salicylic acid was dissolved in 2 ml of 40% ethanol (*solution A*); 0.5 ml of solution A was diluted by 4.5 ml of 40% ethanol (*solution B*); 0.5 ml of solution B was diluted by 4.5 ml of 40% ethanol (solution C); 20 □l of solution C was injected into chromatographic system.

Preparation of Phyto Novo-Sed solution: 0.3 mL of Phyto Novo-Sed extract was diluted by 4.5 ml of 40% ethanol, and 0.2 mL of internal standard solution was added.

Component content was calculated using reference standards according to the following formula:

$$X = m_0 * S_x / V * S_0$$

where X — component concentration in mg/ml, m_0 — weight of reference standard in mg, V — dilution volume, S_x и S_0 - peak areas of the studied component and reference standard respectively. Statistical data processing was performed according to [29].

RESULTS AND DISCUSSION

Identification of extract components, which was performed by previously developed HPLC method [28], revealed marker compounds, which can be used for extract standardization. From the point of view of availability and cost of standard substances, it seems expedient to use the following substances as marker compounds: ascorbic acid for rose hip fruits, chicory and 2-hydroxycinnamic acids (purple coneflower grass), rosmarinic acid (lemon balm grass), hyperozide (hawthorn fruits), chlorogenic and caffeic acids (purple coneflower grass and hawthorn fruits), rutin (rose hip fruits, motherwort grass and lemon balm grass), quercetin (rose hip fruits, motherwort grass). These compounds were also found to be main components of extracts of the corresponding herbal raw materials [8-11, 13–15, 17, 21, 23, 30-35].

Quantification of marker compounds within the optimal range of chromatographic parameters appeared to be possible by performing 2 sequential runs of chromatography on octadecylsilane sorbent with different gradient regimens using a mobile phase, which consists of acetonitrile, tetrahydrofuran, phosphate buffer solution (0.1% H_3PO_4 and 0.02 M KH_2PO_4, pH 3) and dimethylformamide (0,15% v/v).

First chromatography run (gradient type 1, table 1) is characterized by slow elevation of the content of strong eluting agent acetonitrile. Its content increases from 10% to 20% within the first 20 min, and then sharply increases to 90% by 30 min time point. These chromatography conditions are optimized for assay of ascorbic, chlorogenic and caffeic acids and rutin (fig.1). The chromatogram on fig.1 shows that these conditions match well the aim of the chromatographic analysis.

Second chromatography run (gradient type 2, table 1) is performed using the same components of the mobile phase, but is characterized by faster achievement of strong eluting conditions (acetonitrile content 35% by 20 min time point). Afterwards, the content of this strongest eluting component of the mobile phase increases more slowly. These chromatography conditions are optimized for assay of hyperoside, quercetin, chicory, rosmarinic and 2-hydroxycinnamic acids (fig.2). Again, the chromatogram on fig.2 shows that these conditions are optimal for analysis of these components.

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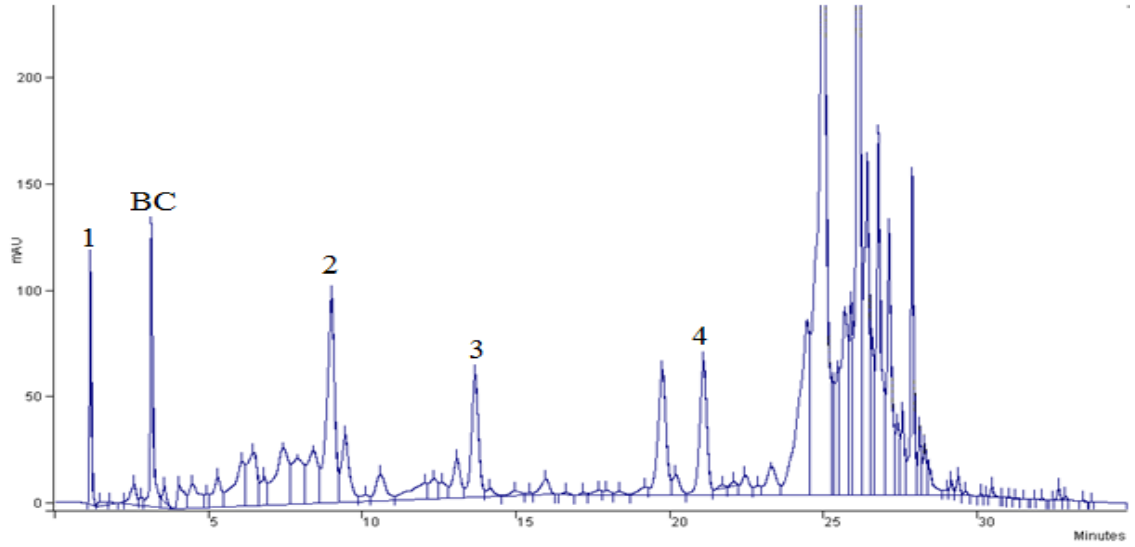

Fig.1. Chromatogram Of Phyto Novo-Sed Sample Using Mobile Phase Gradient 1 (Detection At 285 NM). 1 - Ascorbic Acid, 2 - Chlorogenic Acid, 3 - Caffeic Acid, 4 - Rutin, Bc - Internal Standard (Gallic Acid)

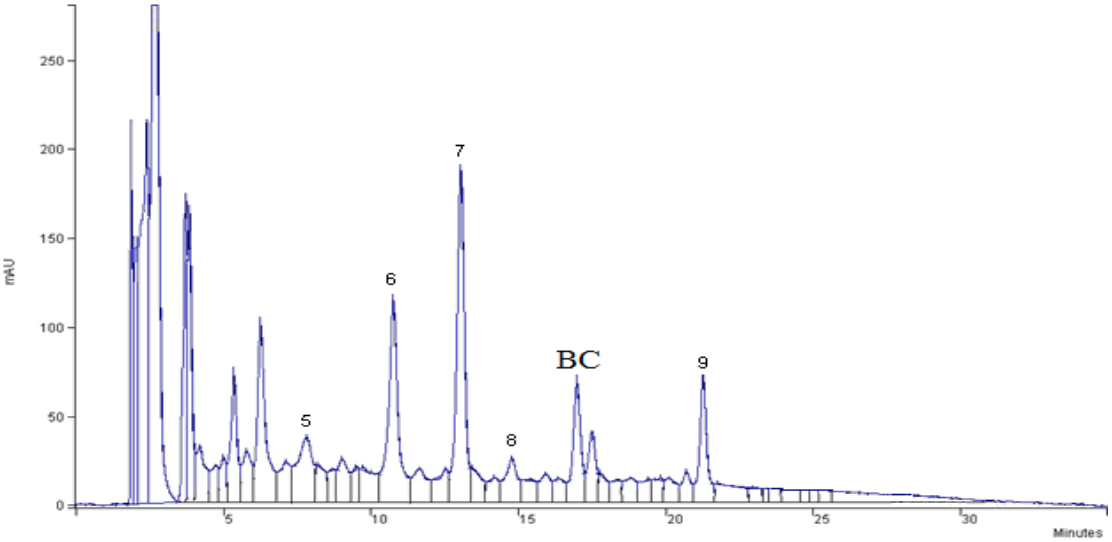

Fig.2. Chromatogram Of Phyto Novo-Sed Sample Using Mobile Phase Gradient 2 (Detection At 285 NM). 5 - Hyperozide, 6 - Chicory Acid, 7 - Rosmarinic Acid, 8-2 - Hydroxycinnamic Acid, 9 - Quercetin, Bc – Internal Standard, Salicylic Acid

Table 1. Regimens of gradient elution during HPLC analysis of Phyto Novo-Sed samples

Time, min	Mobile phase composition, % (v/v)					
	Acetonitrile		Tetrahydrofuran		Phosphate buffer + dimethylformamide	
	gradient 1	gradient 2	gradient 1	gradient	gradient 1	gradient 2
0	10	20	3	3	87	77
10	15	25	3	3	82	72
20	20	35	3	3	77	62
30	90	60	0	3	10	37
35	90	80	0	0	10	20
40	90	90	0	0	10	10

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Table. 2. Parameters of chromatographic separation and quantification of Phyto Novo-Sed marker compounds (Microgen, batch 040414)

[a] – Towards an adjacent peak nearest to the studied one; substances 1-4 – gradient 1; substances 5-9 – gradient 2

№	Substance	Efficacy *N*, theor. plates (n=5)	Selectivity[a] (n=5)	Resolution factor[a] R_S (n=5)	Relative error of peak area value, ε % (n=6)	Content $X_m \pm \Delta x_m$, mg/ml (n=6)
1	Ascorbic acid	18,400	1.42	3.6	3.2	7.76±0.03
2	Chlorogenic acid	24,000	1.64	5.3	6.1	0.92±0.09
3	Caffeic acid	31,400	1.61	2.4	2.7	0.25±0.03
4	Rutin	36,600	1.62	1.3	1.8	0.84±0.07
5	Hyperoside	39,100	1.59	2.7	5.4	0.31±0.03
6	Chicoric acid	23,400	1.27	4.1	2.1	0.51±0.05
7	Rosmarinic acid	28,500	1.12	6.1	5.9	1.10±0.2
8	2-hydroxy-cinnamic acid	23,100	1.24	3.1	1.6	0.14±0.38
9	Quercetin	41,600	1.55	1,7	2.8	0.39±0.03

Table 3. Quantification of marker compounds content (mg/ml) in Phyto Novo-Sed produced by different manufacturers*

COMPOUND	SAMPLE 1	SAMPLE 2	SAMPLE 3	SAMPLE 4	SAMPLE 5
	Microgen		Vatchem Pharmacy		PharmVILAR
	Batch 050414	Batch 060414	Batch 030813	Batch 071213	Batch 010316
Ascorbic acid	5.44	4.69	5.08	5.86	6.04
Chlorogenic acid	0.43	0.50	0.29	0.48	0.57
Caffeic acid	0.14	0.22	0.12	0.31	0.28
Rutin	0.70	0.61	0.46	0.32	0.53
Hyperoside	0.19	0.27	0.16	0.12	0.25
Chicoric acid	0.34	0.45	0.24	0.35	0.39
Rosmarinic acid	1.04	1.09	0.75	0.64	0.83
2-hydroxycinnamic acid	0.14	0.08	0.06	0.03	0.04
Quercetin	0.33	0.42	0.47	0.22	0.28

* mean of 3 independent measurements

Each sample of reference standards and study extract is spiked by internal standard (gallic acid for analysis using gradient 1, and salicylic acid for analysis using gradient 2), which allows more accurate identification of analyte peaks on the chromatogram by relative retention time.

Analysis of UV spectra of reference substances in mobile phase corresponding to time 0 showed that detection is most convenient at wavelengths 285 and 325 nm, where all standards have maximal absorption or a shoulder of the corresponding peak. Marker compounds were identified by comparison of relative retention times (in relation to the corresponding internal standard for the run) and of the ratios of absorbance at 285 and 325 nm for the analytes and reference substances in the same chromatographic conditions.

The proposed method of analysis of the marker compounds by 2 sequential chromatographic runs has been validated for specificity, reproducibility and intermediate precision according to [29]. Suitability of the chromatographic system (parameters) was confirmed both for analysis of study extract (sample of batch 040414 by Microgen) and of model mixtures.

Capacity index k' fell within optimal range 2.1 – 6.2 when using mobile phase gradient 1 and within range 3.7 – 8.9 for gradient 2. Efficacy *N* was not less than 18,000 theoretical plates for each peak, and selectivity α and separation ratio *Rs* for the neighbor peaks were optimal in the model mixture and sufficient in the study extract (table 2).

Specificity of the method is determined by the method ability to quantify marker compounds reliably in the presence of accompanying components of the liquid extract Phyto Novo-Sed. Marker compounds were identified by comparison of relative retention times (in relation to the corresponding internal standard for the run) and of the ratios of absorbance at 285 and 325 nm for the analytes and reference substances. Peaks of marker compounds were well separated from the peaks of accompanying components, and signal/noise ratio exceeded 100 for all studies compounds.

Repeatability of the method is determined by the level of correspondence of the results of individual measurements repeated several times. Table 2 shows repeatability data for the areas of marker compound peaks in Phyto Novo-Sed (batch 040414 by Microgen).

Intermediate precision was measured by analysis of the mixture of reference substances during 3 days in a row, 3 runs a day. Coefficient of variation (CV, %) for 9 samples fell within the range 0.8 – 2.9%.

Thus, repeatability of the peak areas for the model mixture of reference substances as determined by relative error of the mean (relative standard deviation) fell within the range ±3%. Relative error for the corresponding compounds of the study product was 2-fold higher (table 2), but was sufficient for quantification of the marker compounds in this uneasy, multi-component product.

The study confirmed the validity of the developed method by the chosen parameters.

Table 3 shows the results of marker compounds' quantification in the products of different manufacturers using the developed method.

Quantification of the content of marker compounds shows some variation of the results for 3 manufacturers. In all cases, the highest content was found for ascorbic, rosmarinic and chlorogenic acids, as well as for rutin and quercetin.

CONCLUSION

Quantification of Phyto Novo-Sed marker compounds by HPLC is based on 2 sequential chromatographic runs, with gradient regimen of the first run optimized for analysis of more polar substances, and that of the second run – for lees polar substances. The validated parameters show the developed method to be specific and to have correct repeatability, which allows its use for reliable determination of marker compounds in the product. The content of marker compounds (mg/ml) in samples of 6 batches of Phyto Novo-Sed liquid extract produced by different manufacturers falls in the following ranges (in descending order): ascorbic acid (4,7 - 7,8), rosmarinic acid (0,7 - 1,1), chlorogemic acid (0,3 - 0,9), rutin (0,5 - 0,8), chicory acid (0,3 – 0,5), quercetin (0,2 - 0,4), hyperozide (0,1 - 0,3), caffeic acid (0,1 - 0,3), 2-hydroxycinnamic acid (0,06 – 0,14).

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