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Development of a methodology for standardization of herbal drug mixtures

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Abstract:

At present, there is a strong need for the unbiased methods for the assessment of the quality of aqueous extracts obtained from various herbal materials. Due to the lack of methods, the quality of such preparations can be assured primarily by quality assessment of the starting material. Current pharmacopoeial monographs on herbal material include mandatory "Content" section, which was absent in the earlier editions of the State Pharmacopoeia of the Russian Federation. The section prescribes assay of biologically active compounds in the aqueous extract, which can be performed in several ways: by qualitative assessment of a single compound, several compounds, or by assessment of total content of a group of compounds expressed as a single reference compound. The choice of target compound for the assay is stipulated by its extractability and pharmacological (therapeutic) activity. Assay is usually performed using modern instrumental methods, e.g., spectrophotometry, chromatography, etc.

However, if the biologically active constituents of herbal mixture are unknown (which is true for most traditional herbal drugs), then the monographs should rely on identification and assay of compounds which can serve as analytical markers.

The paper describes methodology for choosing analytical markers used in standardization of herbal drug mixtures for which no individual pharmacologically active compounds or a group of compounds have been identified. It relies on quantification of constituents which are present in most of the herbal drug mixtures.

Using proposed methodology, a spectrophotometric procedure for the assessment of total flavonoid content (TFC) of two sedative herbal drug mixtures was developed and validated. The limit of TFC expressed as rutin is proposed.

Keywords: herbal mixtures, herbal drugs, standardization, biologically active compounds

INTRODUCTION

Recent studies have shown that there is a growing trend of psycho-emotional disorders in the world population. Various social, economic, and biological factors (e.g., financial problems, global information overload, chronic tiredness, ecological problems, decreased quality of life, etc.) leads to a distress manifested as easy fatiguability, performance impairment, irritation, psychic tension, changes in mood, habits, anhedonia, unmotivated fears, and sleep disorders. In order to negotiate with these symptoms sedatives are the drugs of choice. An increased interest in sedative drugs is due to possibility of self-treatment, ease of dosing and administration, low number of contraindications and side effects [1-4].

Despite advances in the synthesis of active pharmaceutical ingredients and the development of novel drug products, aqueous extracts of different medicinal herbs are still widely used. Due to a relatively low content of biologically active compounds it is virtually impossible to have an overdose and a complex of active ingredients provides diverse therapeutic activity. Moreover, aqueous extracts are inexpensive and easy to obtain. All of the mentioned benefits enables the use of aqueous extracts in prophylaxis and therapy of chronic neurotic and depressive disorders, including neurovegetative dystonia, general neurotic syndrome accompanied by phobic disorders, neurasthenia, nervousness, and sleep disorders.

Unfortunately, most of the pharmacopoeial quality assessment methods are outdated as they prescribe organoleptic assessment (color, smell, and taste – the latter is used only for non-poisonous and low-potent herbal raw material), determination of aqueous extract pH, and dry residue [5, 6]. Therefore, the quality of water extracts can be assured only by standardization of the starting material (herbal raw material). Current pharmacopoeial monographs on herbal drugs contain "Content" section [7-13], but it was absent in the State Pharmacopoeia of the Russian Federation until 13th edition (included in the mutually harmonized State Pharmacopoeia of the Belarus Republic) [12, 14-19].

A general monograph "Herbal mixtures" [20] describes assay of biologically active compounds which are responsible for the pharmacological (therapeutic) activity of the herbal mixture aqueous extract. Assay can be performed in several ways: using single compound, several compounds, or by assessment of total content of a group of compounds expressed as a single reference compound. The choice of the approach is determined by the extractability of the compounds and the pharmacological activity of

the herbal mixture. Assay is usually performed using modern instrumental methods (e.g., spectrophotometry, chromatography, etc.) which should be stated in the monograph [21].

However, if biologically active constituents of herbal mixture responsible for therapeutic activity are unknown then the monographs should rely on identification and assay of compounds which can serve as analytical markers.

The aim of this study was to develop a methodology for choosing analytical markers that can be used in standardization of herbal drug mixtures for which no individual pharmacologically active compounds or a group of compounds have been identified.

MATERIALS AND METHODS

Commercial lots of "Phytosedan No. 2" (6 lots) and "Phytosedan No. 3" (six lots) sedative herbal mixtures were used in the study.

Pharmacopoeial monographs on the following components of the studied herbal mixtures were investigated using information analysis methods:

- *Valeriana officinalis* L.
- *Melilotus officinalis* L.
- *Origanum vulgare* L.
- *Mentha piperita* L.
- *Leonurus cardiaca* L.
- *Glycyrrhiza glabra* L.
- *Humulus lupulus* L., and
- *Thymus serpyllum* L.

The following reagents were used in the experiment: ethanol 96% (Samarkand SDK), glacial acetic acid (Khimmed), and aluminium chloride (Khimmed). All reagents were analytical grade and were used without further purification. Rutin reference standard (CAS 153-18-4) was obtained from PhytoLab (Germany).

Biologically active compounds content was assayed spectrophotometrically (aluminium chloride complexation reaction) using Cary 60 (Varian, USA) spectrophotometer. GH-252 (AND, Japan) analytical balance was used to weigh samples of herbal mixtures.

Procedure: a sample of the herbal mixture was grinded obtaining particles that can pass through a 1.0 mm sieve. About 0.5 g (exactly weighted) of grinded mixture were added to a 250 ml ground-glass volumetric flask, 100.0 ml of 70% ethanol were added, and the flask was weighed ($\pm$ 0.01 g tolerance). The flask was attached to a backflow condenser and was heated on a water bath for 90 min; its content was occasionally shaken. After that the flask was cooled to a room temperature and weighed once again, followed by adding 70% ethanol to bring the content to an initial mass.

The content was filtered through a paper filter, discarding first 10 ml (*Solution A*).

10.0 ml of *Solution A* were transferred to a 25 ml volumetric flask, 5.0 ml of aluminium chloride 2% ethanol solution were added to the content of the flask and the content was brought to volume with 96% ethanol (*Test solution*).

10.0 ml of *Solution A* were transferred to a 25 ml volumetric flask, 1 drop of glacial acetic acid was added, and the content was brought to volume with 96% ethanol (*Reference solution*),

After 40 min the absorbance of test solution was measured at 415 nm ("Phytosedan No. 2") or 407 nm ("Phytosedan No. 3") in a 10 mm cuvette.

Flavonoid content (X, %) expressed as rutin in a dry herbal mixture was calculated using the following formula:

$$X = \frac{A \cdot 100 \cdot 25 \cdot 100}{248 \cdot 10 \cdot a \cdot (100 - W)},$$

where:

A – absorbance of *Test solution*;

248 – specific absorbance of rutin-$AlCl_3$ complex at 415 and 407 nm;

a – mass of analyzed sample, in grams;

W – herbal mixture moisture content, in per cent.

Procedure validation was carried out by the following parameters: linearity, repeatability, intermediate precision [22, 23].

RESULTS AND DISCUSSION

Current product specification on "Phytosedan No. 2" and "Phytosedan No. 3" sedative herbal mixtures describes determination of the sum of oxidized substances expressed as tannin. However, obtained spectra had maximum absorbance values at the same wavelength as rutin-$AlCl_3$ complex which allows us to express the determined limit of total flavonoid content (TFC) as rutin.

The analysis of the "Content" section of several pharmacopoeial monographs on the components of studied herbal mixtures showed that none of these components are standardized using sum of oxidized substances expressed as tannin as one of the quality characteristics (Table 1).

Analysis of monographs on components of sedative mixtures showed that for most of them (85% and 75%, respectively) flavonoid content is assessed (Table 2). Therefore, flavonoids can be used as a group of marker compound and the standardization of herbal mixtures can be carried out by flavonoid content.

The results of spectorphotometric analysis of herbal mixtures aqueous extracts showed that it is inconvenient to perform direct spectrophotometry due to spectrum overlapping. A compexation reaction betweel flavonoids and aluminium chloride results in bathochromis shift of maximum absorbance from 330-350 to 390-410 nm. Addition of $AlCl_3$ alcoholic solution result in appearance of absorbance maximum (about 407-415 nm) which is the same as for the rutin-$AlCl_3$ complex. Use of aqueous extract without aluminium chloride as a reference solution allows to neutralize effect of colored component of the assay (Figures 1 and 2).

Based on the obtained results (Table 3 and 4) the following optimal extraction conditions were determined for both of the studies herbal mixtures:

- particle size – 1 mm;
- extraction agent – 70% ethanol;
- material:extraction agent ratio – 1:200;
- extraction duration – 90 min;
- complexation reaction duration – 40 min;
- aluminium chloride (2% ethanol solution) volume – 5 ml;
- reference standard – rutin.

Linearity was assessed using 6 levels of rutin concentrations varying from 30 to 200% of the nominal value. A mean of three measurements was taken as the absorbance value. Linearity acceptance criterion is the correlation coefficient: if it is close to 1 then the data set can be described as a straight line. Correlation coefficient should not be less than 0.995; in our study it was 0.997 for "Phytosedan No. 2" and 0.999 – for "Phytosedan No. 3".

Repeatability was assessed using single herbal mixture sample which was assayed 6 times during short period of time using same reagents and equipment. Acceptance criterion was expressed as a relative standard deviation (RSD) value which should not exceed 10%. For "Phytosedan No. 2" RSD = 1.39% and for "Phytosedan No. 3" RSD = 3.44% which means that the procedure has good precision under the same conditions.

Table 1. The results of the "Content" section analysis of monographs on components of "Phytosedan No. 2" and "Phytosedan No. 3" sedative herbal mixtures

Herbal raw	Content of the "Content" section

material	State Ph., XIII ed. [7]	Eur. Ph. [9]	BP [8]	USP [10]	State Ph. Belorus. [11]	State Ph. Khazah. [12]	State Ph. Ukraine [13]
Valeriana officinalis L.	Extractable matter (70 % alcohol); sesquiterpenic acids expressed as valerenic acid	Essential oil; sesquiterpenic acids expressed as valerenic acid	Essential oil; sesquiterpenic acids expressed as valerenic acid	Extractable matter (70 % alcohol); essential oil; sesquiterpenic acids expressed as valerenic acid	Sesquiterpenic acids expressed as valerenic acid; esters expressed as valerenic acid ethylester	Essential oil; sesquiterpenic acids expressed as valerenic acid	Essential oil; sesquiterpenic acids expressed as valerenic acid
Melilotus officinalis L.	Coumarin	Coumarin	Coumarin	-	Coumarin	-	Coumarin
Origanum vulgare L.	Flavonoids expressed as luteoline; volatile oil	Essential oil; sum of the contents of carvacrol and thymol in the essential oil	Essential oil; sum of the contents of carvacrol and thymol in the essential oil	-	Essential oil; flavonoids expressed as luteoline;	Essential oil; sum of the contents of carvacrol and thymol in the essential oil	Essential oil; sum of the contents of carvacrol and thymol in the essential oil
Mentha piperita L.	Volatile oil; Total flavonoid content expressed as luteolin	Essential oil;	Essential oil;	Essential oil;	Essential oil;	Essential oil;	Essential oil;
Leonurus cardiaca L.	Flavonoids expressed as rutin; extractable matter (70 % alcohol);	Flavonoids expressed as hyperoside	Flavonoids expressed as hyperoside	-	Flavonoids expressed as hyperoside; iridoids expressed as harpagide acetate	Flavonoids expressed as hyperoside	Flavonoids expressed as hyperoside
Glycyrrhiza glabra L.	Glycyrrhizic acid	Glycyrrhizic acid	Glycyrrhizic acid	-	Glycyrrhizic acid	Glycyrrhizic acid	Glycyrrhizic acid
Humulus lupulus L.	Flavonoids expressed as rutin; essential oil	Extractable matter (70 % alcohol)	Extractable matter (70 % alcohol)	-	Extractable matter (70 % alcohol)	-	Extractable matter (70 % alcohol)
Thymus serpyllum L.	Flavonoids expressed as luteolin-7-O-glucoside; extractable matter (water); extractable matter (30% alcohol)	Volatile oil	Volatile oil	-	Volatile oil	Volatile oil	Volatile oil

Table 2. Percentage of components in "Phytosedan No. 2" and "Phytosedan No. 3" sedative herbal mixtures

Herbal raw material	Content in herbal mixture, %	Quality characteristics (State Pharmacopoeia XIII ed.)
"Phytosedan No. 2"		
Leonurus cardiaca L. [24]	40	Flavonoids expressed as rutin Extractable matter (70% alcohol)
Humulus lupulus L. [25]	20	Flavonoids expressed as rutin Essential oil
Mentha piperita L. [26]	15	Essential oil Flavonoids expressed as luteolin
Valeriana officinalis L. [27]	15	Extractable matter (70% alcohol) Sesquiterpenic acids expressed as valerenic acid
Glycyrrhiza glabra L. [28]	10	Glycyrrhizic acid
"Phytosedan No. 3"		
Leonurus cardiaca L. [24]	25	Flavonoids expressed as rutin Extractable matter (70% alcohol)
Origanum vulgare L. [29]	25	Flavonoids expressed as luteolin Essential oil
Thymus serpyllum L. [30]	25	Flavonoids expressed as luteolin-7-O-glucoside; Extractable matter (water) Extractable matter (30% alcohol)
Valeriana officinalis L. [27]	17	Extractable matter (70% alcohol) Sesquiterpenic acids expressed as valerenic acid
Melilotus officinalis L. [31]	8	Coumarin

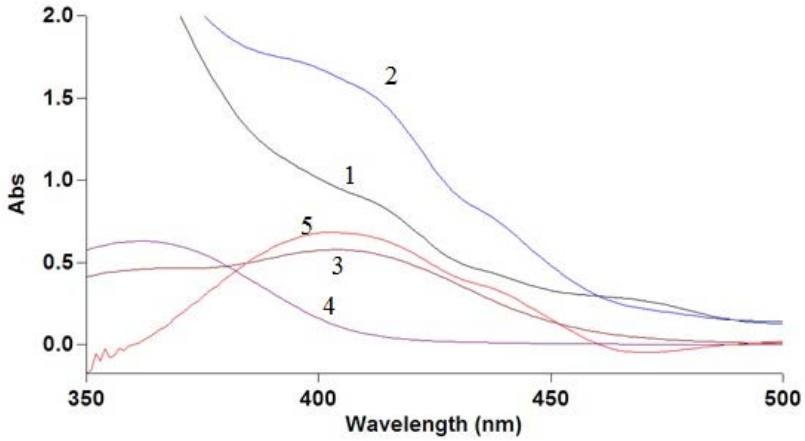

Figure 1. Spectra of "Phytosedan No. 2" alcoholic extracts:
1. Spectrum of "Phytosedan No. 2" alcoholic extract; 2. Spectrum of "Phytosedan No. 2" alcoholic extract plus 2% $AlCl_3$ ethanol solution; 3. Spectrum of "Phytosedan No. 2" alcoholic extract plus 2% $AlCl_3$ ethanol solution against "Phytosedan No. 2" alcoholic extract; 4. Spectrum of rutin reference standard (alcoholic solution); 5. Spectrum of rutin reference standard (alcoholic solution) plus 2% $AlCl_3$ ethanol solution

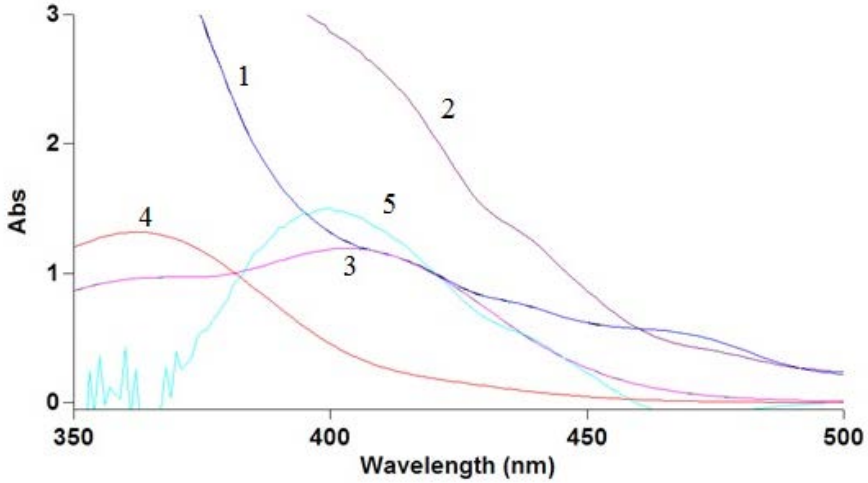

Figure 2. Spectra of "Phytosedan No. 3" alcoholic extracts:
1. Spectrum of "Phytosedan No. 3" alcoholic extract; 2. Spectrum of "Phytosedan No. 3" alcoholic extract plus 2% $AlCl_3$ ethanol solution; 3. Spectrum of "Phytosedan No. 3" alcoholic extract plus 2% $AlCl_3$ ethanol solution against "Phytosedan No. 2" alcoholic extract; 4. Spectrum of rutin reference standard (alcoholic solution); 5. Spectrum of rutin reference standard (alcoholic solution) plus 2% $AlCl_3$ ethanol solution

Table 3. Effect of different extraction conditions on flavonoids extraction

Extraction conditions	Flavonoids expresses as rutin, %	
	"Phytosedan No. 2"	"Phytosedan No. 3"
Particle size, mm		
5-7	0.46	0.78
1-2	0.67	0.93
Ethanol concentration, %		
40	0.42	0.79
70	0.67	0.93
96	0.50	0.52
Material:extraction agent ratio		
1:50	0.57	0.80
1:200	0.67	0.93
1:300	0.67	0.90
Extraction time, min		
30	0.54	0.89
60	0.56	0.91
90	0.67	0.93
120	0.66	0.90

Table 4. Effect of complexation reaction conditions on flavonoid content

Reaction conditions	Flavonoids expressed as rutin, %	
	"Phytosedan No. 2"	"Phytosedan No. 3"
Aluminium chloride (2 % ethanol solution) volume, ml		
1	0.55	0.41
2	0.59	0.50
3	0.65	0.61
4	0.93	0.67
5	0.93	0.67
Reaction duration, mi.		
20	0.61	0.41
30	0.85	0.62
40	0.93	0.67
50	0.93	0.67

Intermediate precision assessment was performed by two chemists on 3 samples in 3 runs. Acceptance criterion was expressed as RSD value which should not exceed 10%. For "Phytosedan No. 2" RSD = 7.37% and for "Phytosedan No. 3" RSD = 4.19%.

Accuracy of the procedure was established by measuring flavonoid content in solutions obtained by addition of different amounts of reference substance (four levels). Acceptance criterion was the mean % recovery which should be within 100 ±5%. For "Phytosedan No. 2" % recovery was within 100.16-102.04% range (mean 100.77%) and for "Phytosedan No. 3" - within 99.40-103.82% range (mean 101.53%).

Obtained results suggest that the procedure is valid.

Analysis of commercial lots of "Phytosedan No. 2" and "Phytosedan No. 3" sedative herbal mixtures using developed procedure showed that flavonoid content expressed as rutin in the former varies from 0.67 to 0.96% which allows us to propose TFC not less than 0.5%, and in the latter – from 0.78 to 1.25%, the proposed TFC not less than 0.6%.

CONCLUSION

Thus, a methodology for choosing analytical markers used in standardization of herbal drug mixtures for which no individual pharmacologically active compounds or a group of compounds have been identified. It relies on quantification of constituents which a present in most of the herbal drug mixtures.

Using proposed methodology, a spectrophotometric procedure for the assessment of total flavonoid content (TFC) of two sedative herbal drug mixtures was developed and validated. Procedure is easily reproducible, fast, and reliable.

The limit of TFC expressed as rutin for two sedative herbal mixtures is also proposed.

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