STUDY OF STABILITY AND DETERMINATION OF RESIDUAL QUANTITY OF ORGANIC SOLVENT IN LIPOPHILIC EXTRACT OF PUMPKIN

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Abstract

Important indicators of medicines' quality are stability and shelf life, that is, the time during which no negative changes in the physico-chemical, pharmacological and consumer characteristics of the product are observed. When developing the composition of a new substance or a drug, the shelf life is determined experimentally, by periodically evaluating all indicators included into the methods of quality control. To establish the qualitative characteristics of the pumpkin extract, the appearance, solubility, identification, microbiological purity, and quantitative content of active substances in the extract were evaluated. To determine the residual amount of organic solvent in the extract, the method of gas-liquid chromatography was used.

Keywords: Stability, lipophilic extract, residual solvent, gas-liquid chromatography.
Introduction

Back in 1959, British scientists Russell W.M.S and Burch Standardization and quality control of medicines in our time remains an important task of modern pharmacy. One of the conditions ensuring the effectiveness and safety of finished drugs is the high quality of active and auxiliary substances used in their manufacture.

Currently, in accordance with the rules of Good Manufacturing Practice 42-4.0: 2008 (GMP), it is the responsibility of manufacturers of pharmaceutical products to conduct a stability study, as a result of which the expiration date and storage conditions of active substances are established. Stability studies should be carried out at the stage of development of pharmaceutical substances or drugs. According to GMP requirements at the development of specifications for raw materials a maximum storage period before re-inspection should be set, and in the specification for finished products there should be shelf life specified. Stability tests are based on obtaining data on the quality change of a substance under the influence of various environmental factors: temperature, light, humidity, etc. The Common Technical Document (CTD) adopted in the EU, USA and Japan establishes the basic requirements for conducting research on the stability of pharmaceutical products (Guidance, 2014; Mitkina et al., 2015; Medvetsky et al., 2014; Lyapunov et al., 2016; Ryzhikova et al., 2014; Sakaeva et al., 2013).

International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) has adopted resolution for the regulation of residual amounts of organic solvents, which establishes the limits of their contents in substances, auxiliary substances and finished pharmaceuticals.

Residual solvents in drugs are volatile organic substances that are used and not completely removed in the process of substances, excipients or finished drugs production.

Since residual solvents have no therapeutic effect, they must be removed to meet the requirements of the specification, good manufacturing practice (GMP) or other quality requirements. Medicines should not contain residual solvents in quantities exceeding the established limit. In the production of active pharmaceutical substances, excipients and drugs use solvents of the following classes of toxicity: having high toxicity - class 1 (benzene, carbon tetrachloride, etc.), less toxic solvents - class 2 (acetonitrile, hexane, methanol, formamide, toluene and others), the safest are solvents of class 3 (anisole, acetone, heptane, etc.).

Thus, all substances and finished drugs should be controlled for the content of those solvents that may be present in them. The norms of the content of residual organic solvents in substances must be justified taking into account the degree of toxicity of each of them to humans and the environment.

In addition, information about the solvent used in the production process of the substance, as well as data on stability should be indicated in the registration dossier of the substance manufacturer, which is submitted during state registration to obtain a certificate of compliance with the Pharmacopoeia (Sabirzyanov et al., 2017; Egorova et al., 2014; Sadchikova et al., 2004).

To control residual solvents of class 1 or 2 (or class 3 with a content of more than 0.5%), if possible, use the procedure described in the general pharmacopoeial monograph or use a suitable validated procedure. In the quantitative determination of residual solvents, the result obtained is taken into account in the quantitative analysis of the substance, except for the cases when the determination of the mass loss during drying is carried out. The concentration limit of hexane in pharmaceutical substances in (ppm) is 290.

In previous works, we have developed a technology for producing a lipophilic extract from pumpkin meal pulp (Cucurbita pepo L. and Cucurbita moschata (Duch) Poir.), which was a homogeneous, oily, resinous mass with a specific odour, orange colour, insoluble in water, ethanol and highly soluble in chloroform, hexane, and ether (Vishnevskaya et al., 2014).

The aim of our work was to study the stability and the shelf life of the lipophilic extract, as well as to determine the residual amount of organic solvent in the lipophilic extract of pumpkin.
Methods

Stability studies were carried out in accordance with the manual 42-3.3: 2004 "Guide to quality. Medicines. Stability study" (Lyapunov et al., 2004).

To establish the shelf life, we observed the samples of the extract for 27 months of storage in dark glass bottles in a dry place protected from light, at temperature conditions 5 ± 3 °C (in the refrigerator) and 25 ± 2 °C. Considering the consistency of the studied samples, as packaging we have chosen glass bottles used for liquid dosage forms in the pharmaceutical industry and ensuring tightness during long-term storage.

The quality control of plant extracts is regulated by the Pharmacopoeia according to the following indicators: organoleptic indicators, identification, quantification, microbiological purity, etc.

In the study of the quality of the lipophilic extract, organoleptic indicators (description) were primarily evaluated. For this, the appearance and characteristic organoleptic properties of the samples (colour, odour, texture), as well as signs of physical instability and solubility, were controlled.

In previous works, we have developed methods for qualitative (identification) and quantitative determination of active substances in the extract, studied the microbiological purity of the extract (Degtyarova et al., 2014; Degtyarova et al., 2015; Degtyarova, 2015; Degtyarova et al., 2015).

Identification of carotenoids was performed by thin layer chromatography (TLC) according to the requirements of State Pharmacopoeia of Ukraine (SPU) 2, art. 2.2.27. Quantitative determination of the number of carotenoids in terms of β-carotene, was performed by the method of absorption spectrophotometry in the visible region (SPU 2, art. 2.2.25). Quantitative determination of the amount of phytosterols in terms of cholesterol was performed by high performance liquid chromatography (HPLC) (SPU 2, Art.2.2.29). Microbiological purity study was carried out according to the method SPU 2, Art.2.6.12. To determine the antimicrobial activity, we used the method of diffusion into agar with reference strains of microorganisms: P. aeruginosa ATCC 9027, S. aureus ATCC 6538 (State Pharmacopoeia of Ukraine, 2015).

To determine the residual amount of organic solvent in the extract, gas-liquid chromatography was used (Cunha et al., 2011; Cicchetti et al., 2008; Egazaryants, 2009; Hadjmohammadi et al., 2016; Hadjmohammadi and Ghoreishi, 2011; Hadjmohammadi and Ghoreishi, 2011; Pervova et al., 2016; Marriott et al., 2009; Usova et al., 2017).

Conditions for analysis:
1. Instrument requirements and chromatographic conditions.
Gas chromatograph with a flame ionization detector, in which the following conditions were set up:
- column: quartz capillary, 60 x 0.32 mm in size, 1.8 μm DB-624 or equivalent, for which the requirements of the "Chromatographic System Suitability Test" are fulfilled;
- the column thermostat temperature was programmed from 40 °C (delay 5 min) to 200 °C (delay 20 minutes), temperature rise - 5 °C / min;
- evaporator unit temperature - 230 °C; flow separation - 1: 5;
- detector temperature - 290 °C;
- velocity of carrier gas (helium) - 2 ml / min.
2. Preparation of working standard sample solution (WSS).
5 ml of the internal standard solution, 12.7 μl of hexane were placed in a 100 ml volumetric flask. The volume was adjusted with the internal standard solution to the mark and mixed (solution 1). 3.0 ml of solution 1 placed in a vessel that was tightly closed (3 samples) with a capacity of 20.0 ml, 0.5 ml of water P, 0.5 g of sodium chloride P and 1.0 g of substance were added. The vessels were immediately sealed with a rubber gasket with a fluoroplastic coating. Then they were alternately placed in a thermostat - a device for analysing the equilibrium vapor phase - and kept at a temperature of 120 °C for 30 minutes.
3. Preparation of the internal standard solution.
100 mg of n-butanol P placed in a 10.0 ml volumetric flask. Brought the volume with a solution of dimethylacetamide (DMAc P) to the mark and stirred. 5.0 ml of the resulting solution placed in a volumetric flask with a capacity of 500.0 ml. Brought the volume of the DMAc P solution to the mark and mixed.
4. Preparation of the solution to verify the suitability of the chromatographic system. 5 ml of the DMAc P solution placed in a 200 ml volumetric flask, 1.5 g (accurate weight) of methanol P, 1.5 g (accurate weight) of acetone P, 0.3 g (accurate weight) of methylene chloride P, 1.5 g (accurate weight) of ethyl ether P, 0.445 g (accurate weight) of toluene P, 0.1 g (accurate weight) of pyridine P were added. Diluted the volume of the DMAc P solution to the mark and mixed.

3.0 ml of the obtained solution placed in a vessel, tightly closed, with a capacity of 20.0 ml, 0.5 ml of water P, 0.5 g of sodium chloride were added. The vessels were immediately sealed with a rubber gasket with a fluoroplastic coating. Then they were alternately placed in a thermostat — a device for analysing the equilibrium vapor phase — and kept at a temperature of 120 °C for 30 minutes.

5. The technique.
1.0 g of the substance placed in a vessel (3 samples) with a capacity of 20.0 ml, tightly closed, 0.5 ml of water P, 0.5 g of sodium chloride and 3.0 ml of an internal standard solution (n-butanol P) were added. The vessels were immediately sealed with a rubber gasket with a fluoroplastic coating. Then they were alternately placed in a thermostat — a device for analysing the equilibrium vapor phase — and kept at a temperature of 120 °C for 30 minutes.

Chromatographed the gas phase over a solution of WSS, obtaining from 2 to 6 chromatograms. The injection volume was 1.0 ml. For the peak areas of the solvents from the chromatograms obtained, the relative standard deviation (RSD) was calculated. The preparation of parallel chromatograms (n0) was stopped when the requirements of the suitability of the chromatographic system were reached (Fig. 4.1, 4.2).

The results were considered reliable if the requirements of the “Test of the suitability of the chromatographic system” were met.

The quantitative content of hexane (X), in ppm, in the sample of the drug was calculated according to the formula:

\[ X = \frac{B_i \cdot 3 \cdot 10^6 \cdot \rho \cdot V_o}{(B_0 - B_i) \cdot m \cdot 100 \cdot 1000} \]

Where:
- \( B_i \) - the average value of the peak area of hexane in the chromatogram of the test solution;
- \( B_0 \) - the average value of hexane peak area in the chromatogram of WSS;
- \( V_o \) - the volume of hexane, µl;
- \( \rho \) is the density of hexane (0.6548);
- \( m \) is the mass of the sample of the drug in grams.

The residual content of hexane in the extract should not exceed 290 ppm.

6. Testing the suitability of the chromatographic system.

The chromatographic system was considered suitable if the following conditions were met:

- the degree of peaks separation, calculated by the peaks of solvents from chromatograms of the solution, to check the suitability of CS, should have been at least 1.5;

- the relative standard deviation calculated for the peak areas of the solvents from chromatograms of SS solution should have been no more than 6.0%;

- the coefficient of peaks symmetry, calculated by the peaks of the solvent from the chromatograms of the solution to check the suitability of CS, should have been from 0.8 to 1.5

Chromatograms of the WSS solution and the test sample solution of the lipophilic pumpkin pulp extract are presented in Figures 1, 2. The data of the peaks shown on the chromatograms of the WSS and the test sample of the lipophilic extract are presented in table 1.

Results and Discussion

As a result of studies, hexane was found in a lipophilic extract - a solvent of class 2 toxicity. The results of the residual solvent content in the extract (in ppm) in the lipophilic extract do not exceed the regulated norms. Thus, the presence in the extract of hexane in an acceptable amount indicates the possibility of its use as a solvent in the technology of obtaining lipophilic pumpkin pulp extract.
When stored for 27 months and at a temperature of 25 ± 2 °C and relative humidity (60 ± 5) %, the extract samples did not change their appearance, but after 6 months of storage the quantitative content of carotenoids and phytosterols in the extract did not meet the requirements of quality control methods.

Based on the obtained results, the stability of the extract for 24 months was proved and the shelf life was determined - 2 years in dark glass bottles at temperatures of 5 ± 3 °C (in the refrigerator) (Table 2, Table 3).

Acknowledgments

The authors are grateful to the National university of pharmacy of the Ministry of Health of Ukraine

References

Table 1. The data of the peaks shown on the chromatographs of the WSS and the test sample of the lipophilic extract

<table>
<thead>
<tr>
<th>Object of study</th>
<th>Retention time</th>
<th>Peak area</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexane</td>
<td>9,516</td>
<td>98635</td>
</tr>
<tr>
<td>Lipophilic extract</td>
<td>9,523</td>
<td>93129</td>
</tr>
</tbody>
</table>

Table 2. Stability indicators of lipophilic extract during storage in dark glass bottles at 5 ± 3 °C in the refrigerator

<table>
<thead>
<tr>
<th>Indicator</th>
<th>Storage period, months</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Start 3 6 9 12 18 24 27</td>
</tr>
<tr>
<td>Appearance</td>
<td>2 3 4 5 6 7 8 9</td>
</tr>
<tr>
<td>Solubility</td>
<td>Orange oily resinous mass with a specific odour</td>
</tr>
<tr>
<td>Identification</td>
<td></td>
</tr>
<tr>
<td>carotenoids</td>
<td></td>
</tr>
<tr>
<td>With a solution of phosphomolybdic acid P</td>
<td>Carotenoids - blue staining</td>
</tr>
<tr>
<td>With a solution of Stibium (III) chloride P</td>
<td>Carotenoids - green staining</td>
</tr>
<tr>
<td>TLC (after treatment with a 5% alcohol solution of phosphomolybdic acid P)</td>
<td>On the chromatogram of the test solution, a stain appears at the level of the stain on the chromatogram of the β-carotene SS solution and the stain with a lower Rf value</td>
</tr>
<tr>
<td>Maximum absorption at λ = 450 (absorption spectrophotometry method)</td>
<td>Complies</td>
</tr>
<tr>
<td>Quantitative content</td>
<td></td>
</tr>
<tr>
<td>Carotenoids in terms of β-carotene, mg (from 9.66 to 10.96 mg)</td>
<td>10.23 ± 0.01 10.24 ± 0.02 10.27 ± 0.01 9.89 ± 0.01 9.85 ± 0.02 9.77 ± 0.01 9.71 ± 0.01 8.51 ± 0.01</td>
</tr>
<tr>
<td>Phytosterols in terms of cholesterol, % (from 1.973 to 2.025%)</td>
<td>2.017 ± 0.001 2.021 ± 0.001 2.005 ± 0.001 2.022 ± 0.001 2.013 ± 0.001 1.986 ± 0.001 1.984 ± 0.001 1.887 ± 0.001</td>
</tr>
<tr>
<td>Microbiological purity: aerobic fungi and anaerobic fungi (total)</td>
<td>&lt;10²</td>
</tr>
<tr>
<td>P. aeruginosa, S. Aureus</td>
<td>No growth</td>
</tr>
</tbody>
</table>

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### Table 3. Stability indicators of the lipophilic extract of pumpkin pulp during storage in dark glass bottles at a temperature of $25 \pm 2$ °C and relative humidity $(60 \pm 5)$% 

<table>
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<th>Storage time, months</th>
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</tr>
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<td><strong>Solubility</strong></td>
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</tr>
<tr>
<td><strong>Identification</strong></td>
<td></td>
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<tr>
<td><strong>Carotenoids</strong></td>
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<tr>
<td><em>P. aeruginosa, S. Aureus</em></td>
<td>No growth</td>
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</table>
Figure 1. Chromatogram of the SS solution: 1 - system peak; 2 - hexane; 3 - internal standard; 4 - DMAc P; 5 - system peak.

Figure 2. Chromatogram of the sample solution of the pumpkin pulp lipophilic extract: 1 - system peak; 2 - hexane; 3 - internal standard; 4 - DMAc P; 5 - system peak.