

# Development of the Method of Identification and Quantitation of Active Ingredients in Suppositories “Phytoprost”

V. Hrytsenko<sup>1</sup>, S. Gubar<sup>1</sup>, O. Ruban<sup>1</sup>, L. Bobrytska<sup>1</sup>, S. Kutsenko<sup>1</sup>,  
T. Germanyyuk<sup>2</sup>, T. Ivko<sup>2</sup>

<sup>1</sup>Department of Industrial Technology of Drugs, National University of Pharmacy, Kharkiv, Ukraine,

<sup>2</sup>Department of Pharmacy, National Pirogov Memorial Medical University, Vinnytsya, Ukraine

## Abstract

**Aim:** Development of the method of identification and quantitation of the active ingredients in “Phytoprost” suppositories for the treatment of prostatic hyperplasia. **Materials and Methods:** Identification and quantitation of sterols in suppositories “Phytoprost” with preliminary received unsaponifiable substances were carried out by gas chromatography using a gas chromatograph Shimadzu GC 14-B in the following configuration: Flame-ionization detector, of capillary quartz column measuring 25 m × 0.25 mm with applied stationary phase of poly[methyl (94)phenyl(5)vinyl(1)]siloxane (SE-54) with a layer of 0.25 microns. **Results and discussion:** Development of the method of identification and quantitation was conducted in accordance with the requirements of the general article 2.4.23 of the State Pharmacopoeia of Ukraine 2.0, p. 194 on the base of the laboratory of pharmacological analysis of the State Company “Ukrainian scientific pharmacopoeia center of the quality of medicines.” Test for sterols consists of two parts: Receipt of unsaponifiable substances in accordance with the requirements of general article 2.5.7 of the State Pharmacopoeia of Ukraine 2.0, p. 214, and determination of the composition of sterols in the unsaponifiable remainder by gas chromatography method in accordance with the requirements of the general article 2.4.23 of the State Pharmacopoeia of Ukraine 2.0, p. 194. Identification of sterols was carried out in the presence of campesterol, stigmasterol,  $\Delta^7$ -campesterol,  $\Delta^5,23$ -stigmasterol, and  $\beta$ -sitosterol. The chromatogram of the test solution should exhibit peaks with such relative retention times: 0.84 (campsterine), 0.88 (stigmasterol), 0.93 ( $\Delta^7$ -campsterine), 0.95 ( $\Delta^5,23$ -stigmasterol), and 1.0 ( $\beta$ -sitosterol). **Conclusion:** The method of identification and quantitation of active substances (plant extracts - the fruits of Palma Sabal, the root of Nettle, and Pumpkin seeds) was developed in the suppositories “Phytoprost” for the treatment of prostate hyperplasia. Identification and quantitation of active substances were carried out according to the content of the sterol fraction by gas chromatography with a preliminary preparation of unsaponifiable substances. It is established the composition of the sterol fraction of the unsaponifiable substances and quantitation of sterols in a single suppository in terms of  $\beta$ -sitosterol.

**Key words:** Gas chromatography, identification, plant extracts, quantitation, suppositories

## INTRODUCTION

Benign prostatic hyperplasia (BPH) is a widespread disease in men with an age-related tendency to progressive growth. The long-term, complex drug therapy is recommended in the early stages. The complex therapy includes herbal preparations and biologically active substances that provide a wide range of therapeutic action, high bioavailability, and safety of use.<sup>[1-3]</sup>

Today, in the world, more than 60% of patients with BPH are treated with plant extracts, among

that the most popular are preparations based on extracts of Palm Sabal (*Sabal serrulata* or *Serenoa repens*). Furthermore, extracts of Dwarf Plum, Chamomile, Nettle root, and Pumpkin seeds widely are used for the treatment of BPH.<sup>[4-7]</sup>

### Address for correspondence:

T. Ivko, Department of Pharmacy, National Pirogov Memorial Medical University, Vinnytsya, Ukraine.  
E-mail: ivkot1981@gmail.com

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The effectiveness of these plants is due to the presence in them of free fatty acids, triterpenes, and phytosterols, especially sitosterols. Mechanism of action of these plants is the suppression of the synthesis of prostaglandins in the prostate, inhibition of globulin production in the liver, which binds sex hormones. They have antimicrobial, membrane-stabilizing, antioxidant, anti-inflammatory, antispasmodic effects, ability to improve hemodynamics and restore sexual function. The most expedient is the development of prostate protective substances in the form of suppositories since this is the most optimal dosage form for the treatment of prostate diseases.<sup>[8,9]</sup> To create a new prostate protector rectal suppositories “Phytoprost” were developed. These suppositories contain a composition of a fruit extract of Palm Sabal, the extracts of Nettle roots and Pumpkin seeds that were introduced into the fatty base.<sup>[10]</sup>

The purpose of the research was the development a method of identification and quantitation of the active ingredients in suppositories “Phytoprost.”

The objectives of the study were a method of identification and quantitation of the active ingredients in “Phytoprost” suppositories. Determination and quantitation of the composition of sterols in the suppositories “Phytoprost” was carry out by gas chromatography with preliminary obtaining of unsaponifiables substances.

## MATERIALS AND METHODS

The samples of suppositories “Phytoprost” were investigated: They had a mass of 2.8 g, and they were made pouring method on the basis of solid fat.

Identification and quantitation of sterols in suppositories “Phytoprost” with preliminary received unsaponifiable substances were carried out by gas chromatography using a gas chromatograph Shimadzu GC 14-B in the following configuration: Flame-ionization detector, of capillary quartz column measuring 25 m × 0.25 mm with applied stationary phase of poly[methyl (94)phenyl(5)vinyl(1)]siloxane (SE-54) with a layer of 0.25 microns.

### Determination of sterols relative to $\beta$ -sitosterol

The process of sterols definition consists of two parts: Unsaponifiable substances obtaining (in accordance with the requirements of general article 2.5.7 of the State Pharmacopoeia of Ukraine 2.0, p. 214) and determination of the sterol composition in the unsaponifiables remainder by gas chromatography method in accordance with the requirements of the general article 2.4.23 of the State Pharmacopoeia of Ukraine 2.0, p. 194.<sup>[11]</sup>

20 suppositories were placed in pounder, and they were pounded to obtain a homogeneous mass carefully. Powdered suppository mass 2.8 g of the substance was placed in a 250 ml flask with a fridge. Then, we added 50 ml of 2 M alcoholic solution of potassium hydroxide R, and we heated it in a water bath for 1 h, mixing periodically. After cooling to room temperature, the contents of the flask were quantitatively transferred with 15 ml of water R to a separatory funnel with a capacity of 500 ml containing 85 ml of water R. After that, we have shaken it within 5 min and we extracted it with ether free from peroxides R 3 times in portions of 100 ml each.

All ether extracts were collected in another dividing funnel, in which 40 ml of water R was previously placed. Further, we gently shook it for 2 min and left until the layers were completely separated, after that the aqueous layer was expelled.

The ether layer was washed with two portions of water R, 40 ml each, then carefully washed it in turn 40 ml of solution of 30 g/l potassium hydroxide R and 40 ml of water P. This procedure was repeated 3 times.

Later the ether layer was washed with water R in portions of 40 ml each so long as the water layer gives an alkaline reaction to phenolphthalein. The ethereal layer quantitatively was transferred in a round-bottomed flask of 100 ml capacity using ether free of peroxides R.

Ether was distilled with appropriate caution and 6 ml of acetone R was added to the residue. The solvent was thoroughly removed in a stream of air.

The flask with the residue was dried at a temperature from 100°C to 105°C to constant mass and cooled it in a desiccator.

Sterols were determined in the obtained unsaponifiables substances. The testings were carried out using gas chromatography method. All operations were carried out by protecting solutions and reagents from moisture, solutions were prepared immediately before use.

### Test solution

To the residue in the flask obtained in the determination of unsaponifiables substances, 100  $\mu$ l of pyridine anhydrous R was added and was stirred until the sample was dissolved. The solution we placed in vial with volume 2 ml and added 100  $\mu$ l of a mixture of reagent Sigma-Aldrich (N,O-Bis(trimethylsilyl)trifluoroacetamide with trimethylchlorosilane) (Sigma № T 6381). Viala was closed, and we kept it for 30 min at 40°C. After that, the solution was evaporated in an inert gas stream at room temperature to dryness (about 30 min), and obtained residue was dissolved in 500  $\mu$ l of cyclohexane R.

## Reference solution

About 0.003 g of  $\beta$ -sitosterol was placed in the Viala with a capacity of 2 ml, and we added 100  $\mu$ l of *pyridine anhydrous R* and stirred until the sample was dissolved. To the solution, 100  $\mu$ l of a mixture of the reagent Sigma-Aldrich (N,O-Bis(trimethylsilyl) trifluoroacetamide with trimethylchlorosilane) (Sigma № T6381) was added. Viala was closed and was kept for 30 min at 40°C. Then, the solution was evaporated in an inert gas stream at room temperature to dryness (30 min). The obtained residue was dissolved in 500  $\mu$ l of *cyclohexane R*.

We chromatographed on a gas chromatograph with a flame-ionization detector under the following conditions:

- Capillary quartz column, 25 m  $\times$  0.25 mm in size, with a stationary phase of *poly[methyl (94)phenyl(5)vinyl(1)] siloxane R* (SE-54) with a layer thickness of 0.25  $\mu$ m, or similar with requirements of the test “Checking the suitability of the chromatographic system;”
- Carrier gas: *Helium for chromatography R* or *hydrogen for chromatography R*;
- Speed of carrier gas – 0.6 mL/min;
- Column temperature – 250°C;
- Temperature of the block of input of samples – 270°C;
- Temperature of the detector – 270°C;
- Separation of the flow 1:40.

We chromatographed in 2  $\mu$ l of the test solution and the reference solution alternately, and we received at least five chromatograms for each of the solutions.

The chromatographic system is considered suitable if the following conditions are met:

- Efficiency of the chromatographic column, calculated on the basis of the  $\beta$ -sitosterol peak on the chromatograms of the comparison solution, should be at least 5000 theoretical plates;
- Coefficient of separation between the peaks of campesterol and stigmasterol on the chromatogram of the test solution should be at least 2.0.
- On the chromatogram of the test solution, the main peak with the retention time should be detected. This main peak coincides with the  $\beta$ -sitosterol maintenance period on the chromatogram of the reference solution.

The identification of the peaks of sterols was performed by a gas chromatography method on the time of sterol retention with respect to  $\beta$ -sitosterol. The retention time of  $\beta$ -sitosterol is taken as a unit in accordance with requirements 2.4.23 State Pharmacopoeia of Ukraine 2.0, p. 214.

## RESULTS AND DISCUSSION

The term “unsaponifiable substances” are applicable to non-volatile substances at a temperature from 100°C to 105°C

that is extracted with an organic solvent from the test sample after it saponification. Phytosterols are plant sterols released from the non-saponifiable part of the lipids of plants. There is a wide class of plant substances structurally extremely close to the animal cholesterol. In the tissues, the sterols are in the Free State and in the form of esters with fatty acids - sterols. Phytosterols are the natural components of cell membranes of plants. The most important phytosterols are  $\beta$ -sitosterol, campesterol, and stigmasterol.

It was detected peaks on the chromatogram of the test solution with the following relative retention times: 0.84 (campesterol), 0.88 (stigmasterol), 0.93 ( $\Delta^7$ -campesterol), 0.95 ( $\Delta^5.23$ -stigmasterol), and 1.0 ( $\beta$ -sitosterol) [Figure 1]. The relative time of retention of sterols is determined with respect to the  $\beta$ -sitosterol peak, the retention time of which is taken as unit. Additional peaks are allowed.

The content of the special sterol ( $X_i$ ) in the sterol fraction of the unsaponifiable residue in percentages was calculated by the formula:

$$X_i = \frac{S_i \times 100}{\sum S_i}, \text{ where}$$

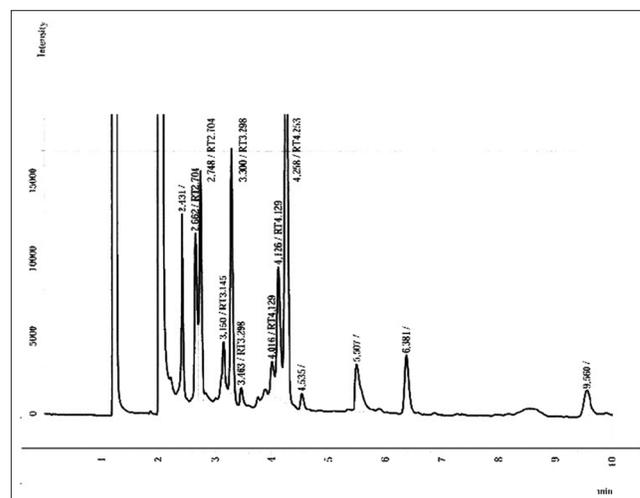
$S_i$  – average value of the peak area of the separate sterol, calculated from the chromatograms of the test solution;

$\sum S_i$  – average value of the areas of all peaks, calculated from the chromatograms of the test solution.

The content of sterols in the sterol fraction unsaponifiable residue should be within range:

campesterol – 5.0–8.0%;

stigmasterol – 10.0–15.0%;



$\Delta^7$ -campesterol – 1.0–3.5%;

$\Delta^5,23$ -stigmasterol – 9.0–15.0%;

$\beta$ -sitosterol – 30.0–40.0%.

Quantitative content of sterols was calculated in two ways: The percentage of each sterol in the sterol fraction (unsaponifiable residue) of the drug, and the sum of sterols in a single suppository in terms of  $\beta$ -sitosterol.

The content of the sum of sterols (Y) in one suppository, in terms of  $\beta$ -sitosterol, in milligrams, was calculated by the formula:

$$Y = \frac{\sum S_i \times m_0 \times b}{S_0 \times m}, \text{ where}$$

$\sum S_i$  – Average value of the areas of all peaks of sterols, ranging from campesterol to  $\beta$ -sitosterol, calculated from the chromatograms of the test solution;

$S_0$  – Average of the peak areas  $\beta$ -sitosterol, calculated with reference solution chromatogram;

m – Weight of the drug, g;

$m_0$  – Weight of  $\beta$ -sitosterol, mg;

b – Average weight of the suppository, g.

The content of the sum of sterols in a single suppository, based on  $\beta$ -sitosterol, should be at least 1.7 mg.

It is established that the main peak of the reference solution corresponds to the time of retention of  $\beta$ -sitosterol [Figure 2].

A result of the study was obtained chromatograms of dry extracts of *Palme Sabal* (1), *Nettle root* (2), *Pumpkin seed* (3), and test solution (4) [Figure 3].

Using the developed identification method and quantitation of active ingredients in suppositories “Phytprost” was carried out, and it was determined that the quantitative content of the sum of sterols in one suppository in terms of  $\beta$ -sitosterol is from 1.7 to 1.9 mg.

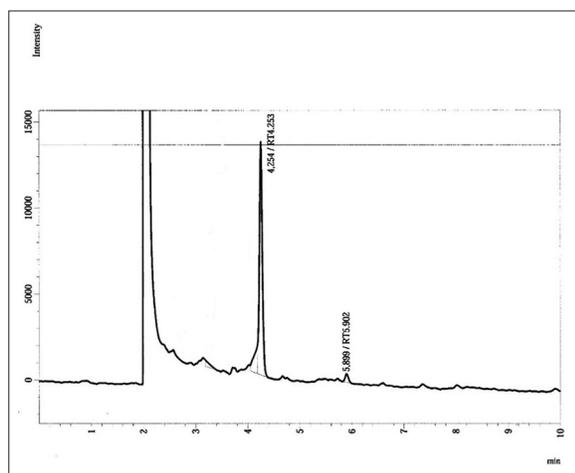


Figure 2: Chromatogram of the reference solution

## CONCLUSION

1. A method of identification and quantitation of active substances was developed (of plant extracts: The fruits of *Palm Sabal*, of the root of *Nettle* and *Pumpkin seeds*) in the suppositories “Phytprost” for the treatment of prostate hyperplasia.
2. Identification and quantitation of active substances were carried out on the content of the sterol fraction by gas chromatography with a preliminary preparation of unsaponifiable substances.
3. The composition of the sterol fraction of the unsaponifiable remainder and the quantitative content

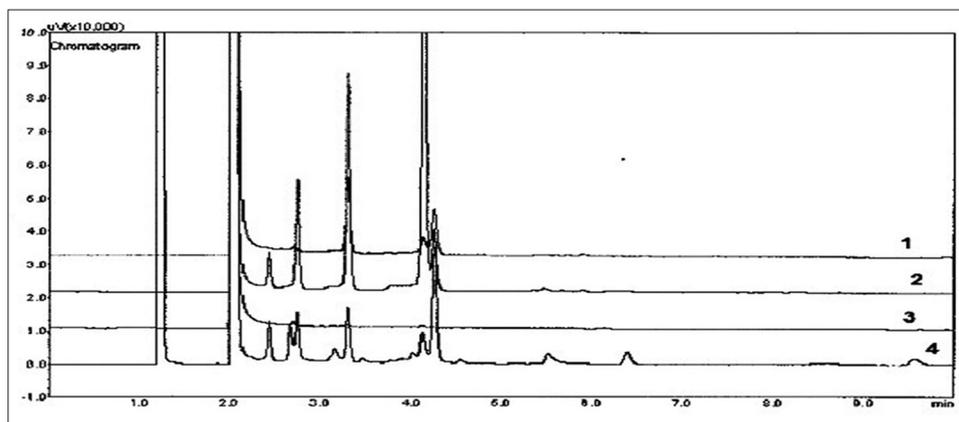


Figure 3: Chromatogram of dry extracts of *palme Sabal* (1), *Nettle root* (2), *Pumpkin seed* (3), and test solution (4)

of the sum of sterols in one suppository in terms of  $\beta$ -sitosterol was determined.

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