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MULTIMERIC HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PLANT STEROLS USING UV DETECTION

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A method for the multimeric high-performance liquid chromatography of plant sterols is proposed which permits the separation of compounds close in structure with similar chromatographic properties. The first stage includes the chemical modification of the sterols with the aid of a bis(p-nitrophenyl) phosphate group. The phosphotriesters formed as the result of the reaction are separated by normal-phase HPLC. The compounds isolated are treated with ammonia, as a result of which the sterol phosphotriesters are converted into the corresponding phosphodiesters. These phosphodiesters are then subjected to reversed-phase HPLC. The chromatographic separation of UV-absorbing sterol derivatives using several variants of HPLC substantially increases the resolving power of the method.

Plant sterols, or phytosterols, form an important field of investigation, since they play a significant role in biochemical processes in plants and are also an initial raw material in the industrial synthesis of hormone preparations. Phytosterols compose a group of structurally related compounds the chromatographic properties of which are very close. This complicates the analysis and preparative processing of individual compounds of this class [1, 2]. Numerous schemes for isolating sterols have been proposed, but the problem still cannot be regarded as having been completely solved [3]. The use for these purposes of such a powerful method of separation as high-performance gas-liquid chromatography (HPLC) is associated with certain difficulties. These are due to the absence of a convenient method of detection, since the majority of sterols contain no chromophoric groups, and also to their low solubility in aqueous organic phases [4]. To circumvent the first of these difficulties it has been proposed to introduce UV-absorbing modifying groups into the sterol molecule. Such a modification is possible, for example, by esterifying the 3-hydroxy groups of sterols [5].

In the present paper we propose a method for the chemical modification of plant sterols with the aid of the bis(p-nitrophenyl) phosphate group which facilitates the detection of these compounds in the process of chromatographic analysis and permits different variants of HPLC to be used for their separation. The triester scheme of synthesis widely known in oligonucleotide chemistry [6] has been used for the introduction of a bis(p-nitrophenyl) phosphate grouping at the 3-hydroxy group of a sterol:

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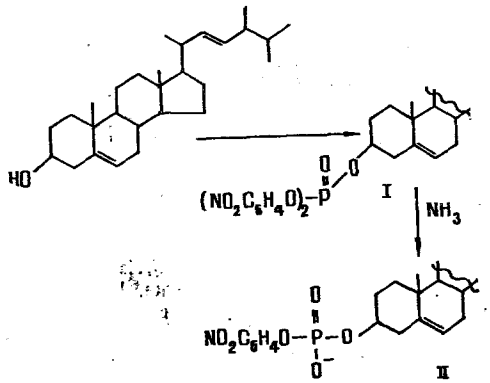

Preparation of UV-absorbing esters of brassicasterol

The addition of this group substantially changes the properties of sterols and facilitates their analysis and preparative processing. In the first place, it becomes possible to detect the compounds under investigation with the aid of a UV detector in the fairly long-wave region (290-310 nm), i.e., to use a mobile phase with a long-wave transmission limit, such as ethyl acetate. In the second place, the addition of the bis(p-nitrophenyl) phosphate group to the 3-hydroxy group of a sterol makes it possible to use a multimeric variant of HPLC. The fact is that the phosphodiesters formed as the result of the modification reaction [structure (I) in the scheme] are readily soluble in various organic solvents, and normal-phase HPLC is excellently suitable for their separation. The performance of an alkaline treatment of the compounds isolated leads to the formation of the corresponding phosphodiesters [structure (II) in the scheme]. In comparison with the initial sterols, these latter compounds possess increased solubility in aqueous phases, so that it is possible to use for the further separation of such compounds the most diverse conditions developed for reversed-phase chromatography. Since such diesters contain the residue of a strong acid, one of the variants of separation may be ion-exchange chromatography or ion-pair chromatography. Thus, by using several variants of separation based on interactions of different nature between sorbent and compounds to be separated it is possible to perform the multimeric separation of the mixture under investigation. This substantially increases the resolving capacity of the method.

The modification reaction is conveniently performed in polypropylene test tubes with lids having a capacity of 1.5-2 ml, which permits the treatment of 8-12 samples simultaneously. In this case, the reaction mixtures are dried by evaporation in a vacuum centrifugal evaporator of the Speed-Vac type (Savant). The modification reaction amounts to treating a sterol with an excess of bis(p-nitrophenyl) phosphate in the presence of an activating agent. After the completion of the reaction, the excess of bis(p-nitrophenyl) phosphate is separated on a silica gel column. In the performance of a preparative synthesis, the excess of bis(p-nitrophenyl) phosphate was illuminated by washing a chloroform extract with water. Then the modification products were subjected to chromatographic separation. Figure 1 shows as an example the separation profile of a mixture of phosphotriesters of brassicasterol (7-dihydroergosterol), ergosterol, and 5-dihydroergosterol. As can be seen from Fig. 1, it is possible to separate such compounds differing only by one double bond.

The conversion of sterol phosphotriesters into phosphodiesters takes place at room temperature and is revealed by the appearance of a yellow coloration of the reaction mixture. The p-nitrophenol split out was extracted with ether; the completeness of extraction was monitored from the disappearance of the yellow coloration of the aqueous layer.

Reversed-phase (rp) chromatography is one of the most popular variants of HPLC. The modifying groups proposed previously permit only a limited choice of conditions for separating sterols by the rp—HPLC method [4]. In view of the increased solubility of sterol diesters in aqueous media, it is possible to use a considerably broader selection of eluents with high water contents. For this purpose, various types of stationary phases have been used: LiChrosorb RP8, Supelco LC8, Zorbax C8.

Figure 2 shows a chromatograph of the separation of a mixture of sterol phosphotriesters on a Supelco LC8 column. As can be seen from Fig. 2, by using a reversed-phase column it is possible to achieve the separation of five sterols structurally extremely close. However, the resolving capacity of the method is increased still further on the successive sepa-

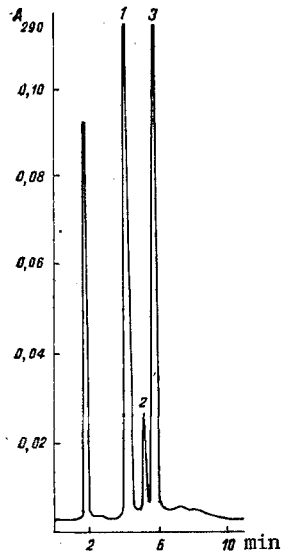

Fig. 1

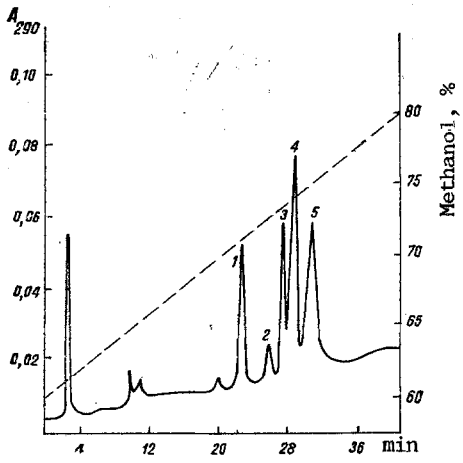

Fig. 2

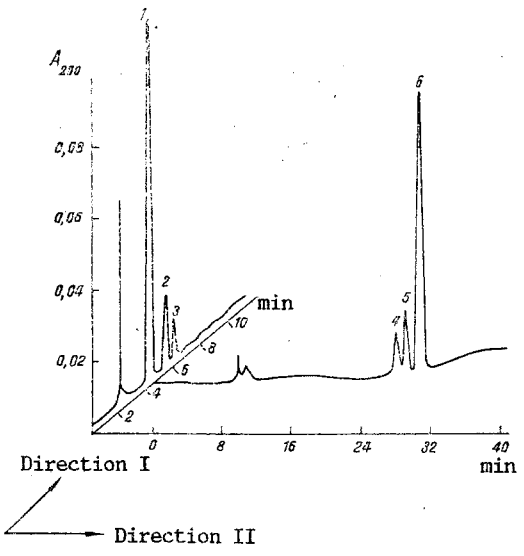

Fig. 3

Fig. 1. Profile of the chromatographic separation of the phosphotriesters of brassicasterol (1), ergosterol (2), and 5-dihydroergosterol (3) on a LiChrosorb Si-60 column (5 μm, 4.6 × 250 mm). Mobile phase: 12.5% of ethyl acetate in hexane. Rate of flow 2 ml/min; temperature 40°C; deposition volume 25 μl.

Fig. 2. Profile of the chromatographic separation of phosphotriesters of ergosterol (1), brassicasterol (2), β-sitosterol (3), stigmasterol (4), and campesterol (5) on a Supelco LC8 column (5 μm, 4.6 × 250 mm) in a concentration gradient of methanol in water (60-80%). Rate of flow 1 μl/min; temperature 40°C; deposition volume 25 μl.

Fig. 3. Two-dimensional chromatography of UV-absorbing derivatives of campesterol, stigmasterol, β-sitosterol, ergosterol, and 5-dihydroergosterol. First direction – chromatographic conditions similar to those given in the caption of Fig. 1. Peak 1) Mixture of phosphotriesters of campesterol, stigmasterol, β-sitosterol; peak 2) ergosterol phosphotriester; 3) 5-dihydroergosterol phosphotriester. Second direction – chromatographic conditions similar to those given in the caption to Fig. 2; peak 4) β-Sitosterol phosphodiester; peak 5) stigmasterol phosphodiester; peak 6) campesterol phosphodiester.

ration of the sterol derivatives first in the form of triesters on a normal-phase column and then in the form of diesters on a reversed-phase column.

Figure 3 shows the result of the two-dimensional chromatography of a mixture of sterols containing ergosterol, 5-dihydroergosterol campesterol, β-sitosterol, and stigmasterol. The sensitivity of detection corresponded to approximately 1 μg of compound in the sample.

Conditions for chromatographic separation in the second direction must be selected on the basis of the presumed composition of the mixture to the separated. Thus, in the case of monohydroxylated compounds one must pass to the reversed-phase variant of separation, and in the study of polyhydroxy compounds the ion-exchange or ion-pair variant of HPLC is possible.

We have used the proposed method of modification for studying not only sterols but also other compounds with a steroid structure and, in particular, the products of the metabolism of anabolic steroid hormones in the animal organism.

EXPERIMENTAL

The work was carried out with ergosterol produced by the Biolar factory (Olaine, USSR); cholesterol, N-methylimidazole, and triisopropylbenzenesulfonyl chloride (TPS) from Merck; ergosterol, brassicasterol, and 5-dihydroergosterol kindly provided by N. A. Bogoslovskii (Vitaminy Scientific Production Combine, Moscow); and β-sitosterol stigmasterol acetate, and campesterol acetate obtained in the phytochemistry laboratory of All-Union Scientific-Research Institute of Medicinal Plants (VNIILR), Mosocw. Pyridinium bis(p-nitrophenyl) phosphate was kindly provided by E. M. Volkov, Lomonosov Moscow State University (MGU im. M. V. Lomonosova), Moscow. TLC was performed on Silufol plates (Czechoslovakia) in the chloroform

(KO) and chloroform–ethanol (9:1) (K10) systems. Free sterols were detected after the chromatograms had been sprayed with a solution of molybdophosphoric acid. HPLC was performed on a SP 8000B chromatograph (Spectra-Physics) fitted with a SP 8440 UV detector (Spectra-Physics) and a WISP 710 (Waters) device for automatic sample injection.

Synthesis of the Sterol Phosphotriester. A mixture containing 1 mg (2.6 μmole) of a sterol and 2 mg (4.7 μmole) of pyridinium bis(p-nitrophenyl) phosphate was placed in an Eppendorf 3812 polypropylene test tube and was dissolved in 200 μl of absolute pyridine, which was then evaporated off in a vacuum centrifugal evaporator. The operation was repeated three times, the contents of the test tube being carefully dissolved in 200 μl of absolute pyridine each time. Then 50 μl of absolute pyridine, 2 μl of N-methylimidazole, and 2 mg (6.6 μmole) of TPS were added to the reaction mixture. The test tube was shaken until the components had dissolved completely and it was then kept in the dark without the access of moisture for 30-60 min. The completeness of occurrence of the reaction was monitored by the TLC method in the KO system.

Then 1 ml of 0.1 M triethylammonium bicarbonate buffer (pH 7.5) was added to the test tube, and it was kept at room temperature for 1 h. The reaction mixture was extracted with chloroform (3 × 1 ml), the extracts were combined and evaporated, and the residue was dissolved in 1 ml of the K10 system. The solution was deposited on a column of silica gel (1 × 2 cm) (Sep-Pak cartridge, Waters) that had previously been equilibrated with 10 ml of K10, and elution was carried out with 5 ml of K10. The combined eluate was evaporated.

Chromatography of Sterol Phosphotriester. The triesters obtained were separated by chromatography on a LiChrosorb Si-60 column (5 μm, 4.6 × 250 mm) in the hexane–10% ethyl acetate or hexane–12.5% ethyl acetate system at a rate of flow of 2 ml/min and a temperature of 40°C. The injection volume was 25-50 μl. The composition of the eluate was monitored by a UV detector at a wavelength of 290 nm. For preparative separation, a LiChrosorb Si-60 (16 × 250 mm) column was used with the same mobile phase at a rate of flow of 50 ml/min. The injection volume was 1 ml.

Preparation of Sterol Phosphodiesters. The fractions that had been collected, containing sterol triesters, were evaporated, the residue was dissolved in 10 μl of pyridine, and 50-100 μl of concentrated ammonia was added. The reaction mixtures were kept at room temperature for 3-16 h and were evaporated to dryness, and each residue was dissolved in 1 ml of water. The aqueous solution was extracted with ether (3 × 1 ml), the ether was blown off in a jet of air or was distilled off in a vacuum centrifugal evaporator. Before injection into the chromatograph, the sample was centrifuged.

Chromatography of the Sterol Phosphodiesters. A 25- to 50-μl sample containing 1-10 μg of the compounds to be analyzed was injected into a Supelco LC8 (5 μm, 4.6 × 250 mm) column, and separation was carried out at the rate of 1 ml/min and a temperature of 40°C in a concentration gradient of methanol in water (60-80%) for 40 min.

CONCLUSIONS

1. A method is proposed for the chemical modification of sterols with the aid of a bis-(p-nitrophenyl) phosphate group.

2. Conditions have been selected for the analysis of the sterol phosphotriesters formed as the result of the modification process by normal-phase HPLC using UV detection.

3. Conditions have been selected for the quantitative conversion of the sterol phosphotriesters into phosphodiesters, and also conditions for the chromatographic analysis of the latter by reversed-phase HPLC.

4. The chromatographic separation of UV-absorbing sterol derivatives using a number of variants of HPLC substantially increases the resolving power of the method and permits structurally close compounds to be separated.

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SPECTROPHOTOMETRIC DETERMINATION OF PAPAVERINE HYDROCHLORIDE BY REACTION WITH PHLOXIN

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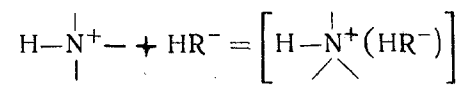
The optimum conditions for the formation of a complex of papaverine hydrochloride with phloxin have been determined: pH 2.5-3.2. A procedure has been developed for the spectrophotometric determination of papaverine hydrochloride. The sensitivity of the determination is 0.4 μg/ml.

Papaverine [1-(3,4-dimethoxybenzyl)-6,7-dimethoxyisoquinoline] is present in opium. Papaverine hydrochloride (I) is widely used in medicine as a spasmolytic drug [1]. The quantitative determination of (I) in pharmaceutical preparations (papaverine hydrochloride, papaverine hydrochloride tablets) is carried out by titrimetric methods [2]. The procedure is distinguished by a high accuracy, but it requires considerable amounts (0.3-0.5 g) of the preparation for analysis. Procedures have been described for the extraction-photometric determination of (I) [3-5], the main disadvantages of which are their lengthiness and the necessity for working with harmful organic solvents.

Our aim was to develop a fairly sensitive procedure simple in use for the quantitative determination of (I) by converting it into a colored compound. It was found that with the organic reagent phloxin (PL), (I) forms an intensively red-violet-colored complex compound. The optical density of solutions of the complex of (I) with phloxin depends on the pH and on the concentrations of (I) and of the reagent. Analysis of the absorption spectra of (I)–PL solutions containing various amounts of (I) showed the possibility of using the complex-forming reaction for the quantitative determination of (I).

The maximum value of the optical densities of solutions of the complex are reached after 3-5 min and then remain constant for not less than 5 h. The optical reaction conditions have been established: temperature 15-30°C; pH 2.5-3.2; 1.5-2.0 ml of a 0.076% solution of phloxin and 1-2 ml of a 0.1% solution of polyvinyl alcohol in 25 ml.

The maximum of the absorption spectrum of the complex lies in the 542-547 nm region. The molar extinction coefficient is $5.33 \cdot 10^4$. The ratio of (I) to PL in the complex has been established as 1:1 by the methods of physicochemical analysis (isomolar series, equilibrium shift). Since the pK_1 and pK_2 values of phloxin are, respectively, 2.41 and 3.13 [6], in the pH 2.4-3.1 interval the singly-charged phloxin anion is present. Under the conditions of interaction with phloxin, (I) is present in solution in the protonated form. On this basis it is possible to give the following reaction equation for the formation of the complex of papaverine hydrochloride with phloxine:



Papaverine hydrochloride interacts with the eosin form of the phloxin molecule dissociated at the hydroxy group symmetrical to the carbonyl oxygen. The interaction of (I) at the carboxy group of phloxin is unlikely, since its π-electronic system is isolated and the ioni-

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