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DETERMINATION OF STICHOPOSIDES IN THE BODY OF THE HOLOTHURIAN *Stichopus japonicus*

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A method has been developed for the quantitative determination of triterpene glycosides — stichoposides — in the tissues of the Far Eastern holothurian *Stichopus japonicus* Selenka. The quantitative determination of the combined triterpene glycosides was based on the isolation of a glycosidic fraction from an ethanolic extract on a column of Polikhrom-1 with subsequent spectrophotometry at 268 nm of the product formed as the result of the reaction of the glycosides with 1 N NaOH. The quantitative determination of the individual glycosides is based on the densitometry of slides or thin-layer chromatography in silica gel with the aid of a Lyuman-IUF-1 luminescence microscope.

The structures of the triterpene glycosides of marine Echinodermata have been studied fairly fully [1, 2]. However, the absence of specific methods for their quantitative determination and the difficulties in obtaining derivatives for gas–liquid chromatography and in separating homologs with the aid of liquid chromatography — all this is interfering with the solution of the main problem of understanding the physiological role of the triterpene glycoside in the producing organisms. In the present paper we discuss a new methodological approach to the quantitative estimation of the individual stichoposides in the tissues of a Far Eastern holothurian.

The tissues of the holothurian *Stichopus japonicus* Selenka contain three triterpene glycosides: stichoposides A, B, and C, which differ from one another by their carbohydrate moieties. It is impossible to separate stichoposide A from stichoposide C by thin-layer chromatography [2], stichoposide C (holotoxin C) being present in trace amounts. In early

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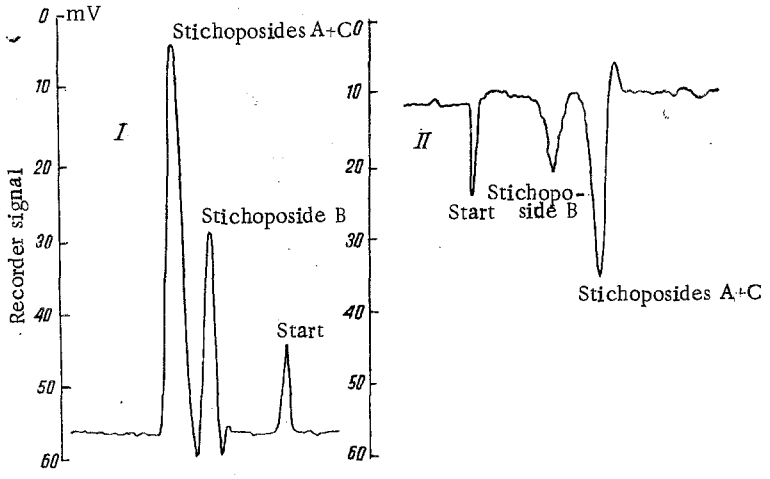

Fig. 1. Densitograms obtained by scanning thin-layer chromatograms of the combined stichoposides (4 μg) by the transmission method: I) Merck silica gel plate; II) negative of a plate coated with silica gel L 5/40 μm.

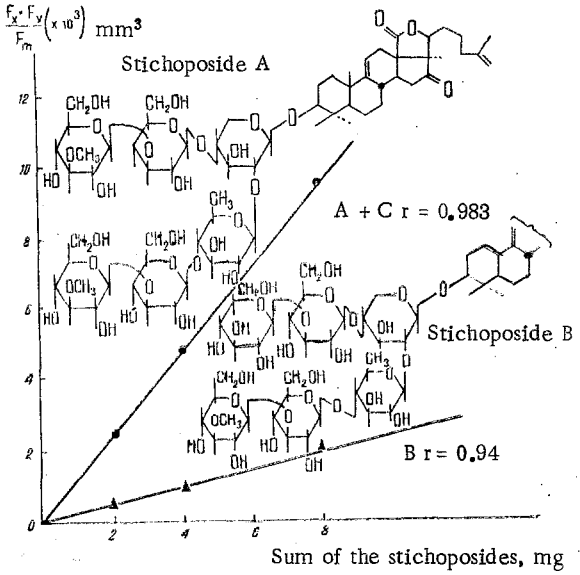

Fig. 2. Calibration graph for detecting the sum of the stichoposides and the structure of stichoposide A and B [10].

studies on the quantitative determination of the triterpene glycosides in holothurians, as a rule, methods based on their hemolytic activity were used [3-5]. This was generally preceded by a lengthy procedure for the extraction and purification of the glycosidic fraction. The production of a pure fraction of the total stichoposides is considerably simplified by using column chromatography with Polikhrom as sorbent. The gradient elution of the stichoposides bound to the Polikhrom permits the isolation of a fairly narrow fraction of glycosides which can be used directly for quantitative estimation. The method is based on the capacity of the stichoposides under alkaline conditions for forming, by the opening of the lactone ring of the aglycone, a conjugated ketone chromophore having an absorption maximum in the UV spectrum at 268 nm [2, 6]. The losses of glycoside connected with breakthrough during the deposition of the extract do not exceed 10%, on average.

The analysis of samples of tissues of the musculocutaneous sac of the holothurian *Stichopus japonicus* collected in the spring period showed that the amount of combined stichoposides was about 0.1% of the weight of the raw tissue, which does not contradict information in the literature [7].

Since in TLC on plates coated with silica gel it is impossible to separate homologous glycosides completely, during the experiment the ratio of the amounts of stichoposides A + C to stichoposide B was estimated.

The process of detecting the spots is associated with the greatest errors in quantitative determination by densitometry. To avoid nonuniform staining we used the dipping of plates coated with Merck silica gel into a 10% solution of H_2SO_4 in ethanol, or the photography of plates prepared from silica gel $L_{5/40}$ μm was followed by the scanning of the negative slides, since in densitometry such plates give considerable "optical noise": The background/signal ratio is very high because of the nonuniformity of the silica gel. Scanning was carried out in the direction of the running of the chromatograms (Fig. 1), and then in the perpendicular direction with adjustment of the elliptical spots of the stichoposides. The accuracy of the determination of stichoposides was determined by repeated photometry of the separated spots of a standard mixture of stichoposides with a known concentration (4 μg):

	Measurement of the height of the peak	
	Stichopisides A + C	Stichoposide B
Number of determinations	12	12
Mean value, mm	140.8	94.2
Maximum and minimum values, mm	149.0—133.0	109.0—60.0
Standard deviation	4.8	15.2
Standard error	±1.02	±3.23

When photography and the scanning of the negatives was used, the intensity of the transmitted light fell somewhat, but the quality of the chromatograms improved because of the decrease in the "optical noise" in the detection of the slides. For samples containing 4 μg of substance the standard deviations ranged from ±3.4 to ±16% for stichoposides A + C and B, respectively, and the linear correlation coefficients for the calibration curves of stichoposides A + C and B were 0.983 and 0.924, respectively (Fig. 2). The ratio of the amounts of stichoposide A + C to B over the range of concentrations studied remained constant at 4.3 ± 0.28.

EXPERIMENTAL

The holothurians were collected in May-June in Alekseev's Bight (Popov Island, Peter the Great Bay) at a depth of 5-8 m. Individuals of both sexes with a weight of the musculocutaneous sac of 140-200 g were taken for analysis.

Extraction. Comminuted pieces of musculocutaneous sac weighing 50 g were extracted three times with 70% ethanol on the boiling water bath. The combined extract was filtered and evaporated to a dry residue, which was redissolved in 10 ml of 70% ethanol.

Column Chromatography. Polikhrom-1 was equilibrated in ethanol for several hours. A 1.5 × 15 cm column was filled with a suspension of the Polikhrom in ethanol and before use it was washed with distilled water until the ethanol had been completely eliminated. A 2-ml portion of the extract was diluted with water to 200 ml and passed through the column at the rate of 5 ml/min, and then the column was washed repeatedly with water. The sum of the stichoposides was eluted with 40% ethanol by volume in 40 ml. The process was monitored by TLC on plates coated with silica gel in the chloroform—methanol—water (65:25:4) system, the spots being revealed by sulfuric acid with heating. The column was regenerated with ethanol.

Quantitative Determination of the Sum of the Stichoposides. For analysis, 1 ml of the fraction containing the combined stichoposides was transferred into a test tube, 1 ml of 2 N aqueous NaOH solution was added, and the mixture was vigorously shaken and placed in the boiling water bath for 10 min. After cooling, the extinction of the solution was measured at 268 nm [6]. As control was used eluent to which an equal amount of water was added in place of the alkali. For calibration a standard sample of the combined glucosides isolated from the holothurian *Stichopus japonicus* was taken [7].

Densitometric Determination of the Stichoposides. For the chromatographic separation of stichoposides we used 5 × 7 cm Merck ready-prepared silica gel plates. The samples were deposited in the form of spots with the aid of a microsyringe of the Hamilton type having a capacity of 1 μl. Separation was performed in the chloroform—methanol—water (65:25:4) system. To reveal the spots, the plates were immersed in a solution containing 10 ml of H_2SO_4 and 90 ml of ethanol, and they were then heated at 160-180°C for 3 minutes, which led to the

appearance of a violet coloration of a white background. A Lyuman-IUF-1 luminescence microscope was used as the detecting instrument.

The chromatograms were scanned in transmitted light, the source of radiation being a DRSh-250-3 lamp with a SZS-24-4 filter. The rate of scanning was 1 mm/sec, and the diameter of the photometering probe 0.5 mm. To plot the calibration curves a proportionality was found between the amount of substance (q) and the expression $\frac{F_x \cdot F_y}{E_{max}}$, where F_x and F_y are the areas under the absorption curves obtained in measuring along the axes of an elliptical spot. E_{max} is the absorption at the common vertex of the curves. The areas under the curves were found by multiplying the height of the peak by its width at half-height. The calibration curve was plotted on the basis of the detection of chromatograms in the range of 1-10 μg of the standard sample of combined stichoposides, and the ratio of the amounts of stichoposides A + C to B was found.

Preparation of the Plates. To separate the stichoposides we also used plates with silica gel $L_{5/40}$ (Chemapol) prepared by a known method [8]. The spots were revealed by spraying the plates with 10% H_2SO_4 in ethanol followed by heating. The chromatograms were photographed on Mikrat-300 film and the negatives were scanned in transmitted light with a red filter, with the determination of the above-mentioned parameters.

The standard deviation, the mean square error, and the correlation coefficients were found from a formula given in the literature [9].

SUMMARY

A method has been developed for the quantitative determination of the combined triterpene glycosides in the tissues of the Far Eastern holothurian *Stichopus japonicus*. It has been found that the amount of the combined stichoposides varies around 0.01% of the weight of the raw tissue.

A method is proposed for the densitometric determination of the stichoposides separated in a thin layer of silica gel.

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