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# Study of *Ukrain* composition using HPLC and UV-spectroscopy methods

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**Summary.** The composition of an antitumor and immunomodulatory preparation *Ukrain* was investigated using HPLC and UV spectroscopy methods. The preparation was found to contain not only already known *Chelidonium majus L.* alkaloids (about 10), but also some substances with non-identified structure. The amount of these substances can be estimated as around 40 %. They are not alkylated alkaloids, because sulfur and phosphorus are not detected in preparation, and they also cannot be attributed to any of the known celandine alkaloids according to their UV-spectra. In composition of *Ukrain* there are no substances proper to the formula declared for it containing three molecules of chelidonine per one molecule of thiotepa (N,N',N''-triethylenethiophosphoramide).

**Keywords:** *Ukrain*, *Chelidonium majus*, alkaloid, thiotepa, celandine.

**Introduction.** *Ukrain* is widely used for malignant neoplasms treatment. It was described as *«a semi-synthetic derivative of Chelidonium majus alkaloids and thiophosphoric acid»* [1]. *Ukrain* is produced by treatment of *Chelidonium majus L.* (*C. majus*) alkaloids with alkylating agent thiotepa. Thiotepa has also been used independently in the chemotherapy of oncological diseases [2]. The authors propose a structural formula for *Ukrain*, which includes a molecule of thiotepa, whose aziridine groups alkylate three chelidonine molecules (Fig. 1).

The results of *Ukrain* investigation performed have been reported in medical literature and in materials of the international congresses on chemotherapy of various diseases [3-5]. They focus mainly on clinical trials of the preparation as an effective antitumor drug, but at the same time its influence on different human organs and systems is being studied in detail (see [6-13] and references therein).

The exception is paper [14] presenting the study on *C. majus* alkaloid mixture used to obtain the preparation. However, the data on *Ukrain* composition are absent both in this article and in other scientific literature. At the same time, there are some publications, the authors of which indicate that the structure of *Ukrain* does not correspond to the claimed formula.

The authors of paper [15], who studied biological activity of *C. majus* alkaloid mixture and *Ukrain* ampoule, failed to find any essential distinctions in their effect on growth and morphology of normal and malignant cells. Considerable

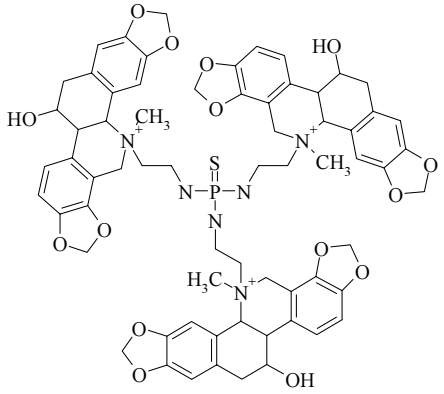

*Fig. 1. The proposed structure of Ukrain.*

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selectivity of both studied objects relative to malignant cells was not revealed either.

The chemical analysis of *C. majus* alkaloid mixture and two samples of *Ukrain* (in ampoule and powder form) by HPLC and LC-MS methods showed that the preparation did not contain the substances corresponding to the suggested structure. It thus seems that *Ukrain* ampoule and *Ukrain* powder contain a significant amount of non-complexed chelidonine [16]. The authors of paper [17] showed that *Ukrain* consists of *C. majus* alkaloid mixture, mainly protopine, chelidonine, allocryptopine and some sanguinarine and chelerithrine.

In the present study, two fractions showing similar retention time on the column and the same UV absorption spectra, alongside with the above mentioned alkaloids, were detected in the composition of *Ukrain*.

According to their spectra, the contents of fractions cannot be referred to any of the known *C. majus* alkaloids. Together they present about 40 % of the total composition. The latter fact could provide the supposition that these are the alkaloids alkylated by thiotepa, but fraction substances did not show positive reactions for sulfur and phosphorus.

**Materials and methods.** The batch (lot number 290614) of *Ukrain* (5 ml ampoule, 1 mg/ml concentration) was used. The classical HPLC, UV spectroscopy, and thin-layer chromatography (TLC) methods were applied in the current work. HPLC was conducted using chromatographic system (Bio-Rad, USA) with flow UV detector model 1306 of UV-monitor type, in reverse phase mode on Bio-Sil ODS-5S column (4x150 mm) at elution rate 0.7 ml/min, in the gradient 0-80 % of isopropanol in 0.05 M sodium phosphate buffer, pH 6.8; 0.2-0.3 ml samples were applied. UV spectra were recorded on Specord UV-VIS (*Karl Zeiss*, Germany).

Berberine, chelidonine, and sanguinarine were used as standard samples. Berberine was isolated from the liana of *Coscinium usitatum Pieere* (*Monispermaceae*). Chelidonine and sanguinarine were kindly provided by L.Yu. Osip (Institute of Animal Biology UAAS, Lviv, Ukraine). The plates of Silufol$^{R}$ UV-254 (Czech Republic) and solvent system methanol — 25 % ammonia — water (5:1:4) were used for TLC. The presence of phosphorus in preparations was determined by spraying the chromatographic plates with detecting agent inclusive ammonium molybdate. The phosphorus containing substances were detected as dark-blue spots on a white background.

The method of sulfur detection is more accurate, but requires more substance for the analysis. 15-20 mg of the material is melted in a test tube with a small piece of metallic sodium (30 mg) in the gas burner flame to red incandescence, whereupon the tube is quickly dipped in another test tube with cold water (4-5 ml). The tube was split. The glass was filtered off, then 2-3 drops of the concentrated aqueous solution of sodium nitroprusside were added to the liquid. In the presence of sulfur the solution becomes dark-violet.

**Results and discussion.** As we showed before [18], in the presence of any donor of protons, trifunctional alkylating agent thiotepa forms active immonium cation Im+, capable of alkylation of nucleophilic sites of 1-3 molecules of the bases. Under acidic conditions (pH<4.5) the breaking of C-N bonds occurs with a formation of alkylated derivatives (Fig. 2).

The atoms of nitrogen and/or oxygen in compounds of chelidonine and protopine groups can serve as nucleophilic sites of alkaloids (weak natural bases) whereas other alkaloids contain only positively charged nitrogen (Fig. 3).

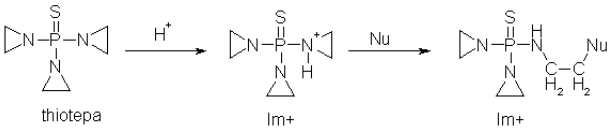


*Fig. 2. Structure of thiotepa, immonium cation (Im+) and alkylated nucleophile (Nu).*

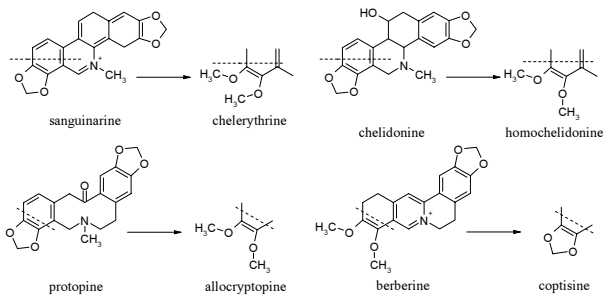


*Fig. 3. Most common alkaloids of Chelidonium majus L.*

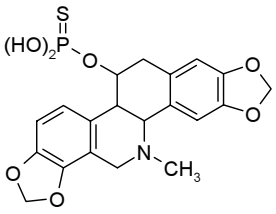


*Fig. 4. Structural formula of Ukrain according to [19].*

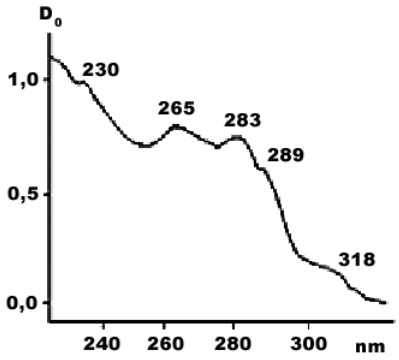


*Fig. 5. UV absorption spectrum of Ukrain.*

This matches data of the paper on the synthesis of different labeled derivatives of chelidonine [19]. In all cases the reaction takes place on the oxygen atom. Authors propose another structure for *Ukrain* (Fig. 4). Moreover, berberine derivatives were synthesized by the introduction of various aromatic groups at C-13 position [20].

In our research UV spectrum of *Ukrain* has two well-marked maximal absorbtion at 265 and 283 nm and three weak peaks at 230, 289, and 318-320 nm area (Fig. 5). At 230 and 265 nm area berberin, coptisine and corisamine absorb, but their inherent intense absorption peak at 347 nm is absent in the preparation spectrum, *i.e.* they are not the main contributors to the total spectrum. Chelerythrine, sanguinarine and allocryptopine have the maximal absorption at 230 and 283 nm, and homochelidonine, chelidonine and protopine — at 288-290 nm. All of them, except for homochelidonine, are also listed in the paper [17]. However, chelidonine and protopine being the major components of *C. majus* alkaloids (their content in the celandine roots is about 60 and 20 % of total alkaloids, respectively [21]), are represented in the preparation spectrum more weakly than other components, in particular those absorbing at 265 nm, as can be seen from the Fig. 5.

Reverse phase HPLC of *Ukrain* and standard alkaloids mixture (berberine, chelidonine, and sanguinarine) showed that the alkaloids from [17] are not the main ones in *Ukrain* composition (Fig. 6, curves 1 and 2). Fractions II and III comprise about 40 % of the preparation. According to chromatography data, the total area of these two fractions peaks was 42.6 %. In the other experiment where the recording was conducted at another wavelength (285 nm instead of 260, Fig. 6) this value was 38 %.

Among the six basic fractions the preparation has been divided into, the UV absorption spectra were invariable only for fractions II and III. Inequality of absorption spectra for other fractions may be explained by the heterogeneity of fraction compositions, when the spectra of the alkaloids mixture, but not individual alkaloids, were recorded.

The separation of standard mixture of alkaloids under the same chromatography conditions showed that fractions V and VI, where at least two products are presented, contain chelidonine and sanguinarine, respectively. The second substance of fraction V is probably homochelidonine, and that of fraction VI — chelerythrine.

The structures of alkaloids of these fractions are similar, therefore, these alkaloids are accompanied by each other (under certain conditions they transform from one to another). Sanguinarine and chelerythrine are often obtained together (so-called *sanguirythrine*) because of the difficulty in their separating.

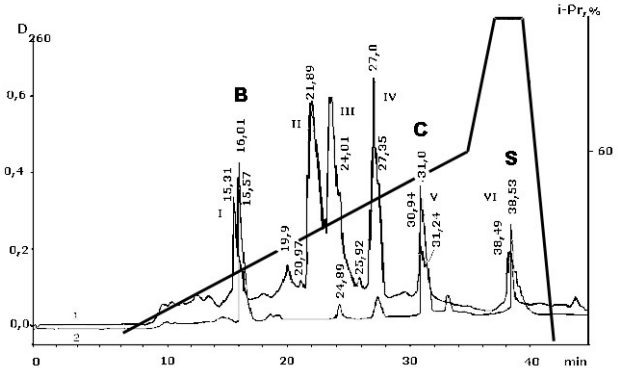


*Fig. 6. Reverse phase HPLC of Ukrain preparation (curve 1) and standard alkaloids mixture (curve 2) on Bio-Sil ODS-5S column (4x150 mm). B, C, and S — berberine, chelidonine and sanguinarine. Eluent: gradient 0-80 % isopropanol in 0.05 M sodium phosphate buffer, pH 7.0; elution rate 0.7 ml/min, 0.25 ml sample of the preparation was applied.*

*Table 1*

*Chromatographic and spectral characteristics of Ukrain fractions and their alkaloid components*

| t, min | Fr. No | $\lambda_{max}$, nm | | Assumed alkaloids |
|---|---|---|---|---|
| | | Experimental data | Literature data and reference | |
| 15.315 | I | 238, 290 | 240, 289 [22] | (chelamine) |
| 15.573 | | 232, 285 | 238, 284 (287) [22] | (chelamidine) |
| 16 | | 229, 265, 346, 420 | 231, 265, 345 [23]<br>228, 265, 347, 420 ** | berberine |
| 21.889 | II | 265 (283), 316 | – | not identified |
| 23 | | 230, 242, 267, 350, 457 | 229, 244, 267, 353, 460 [24] | coptisine |
| 23.216 | III | 265 (283), 314 | – | not identified |
| 24.013 | | 265 (283), 324 | | |
| 26.996 | IV | 233, 285 | 232, 284 [23] | allocryptopine |
| 27.353 | | 239, 290 | 239, 291 [23] | protopine |
| 30.942 | V | 240, 290 | 238, 288 [24]<br>240, 290 ** | chelidonine |
| 31.237 | | 230, 287 | 229, 286 [22] | homochelidonine |
| 38.208 | VI | 229, 284, 320 | 228, 283, 320 [24] | chelerytrine |
| 38.491 | | 240, 285, 330 | 235, 284, 322 [24]<br>240, 285, 330 ** | sanguinarine |

*Note: t — retention time. ** — Our data for standard samples of alkaloids. — The alkaloids forming the fraction of so-called methoxychelidonine [14] are mentioned in the brackets (see text).*

Protopine and allocryptopine, as the basic component in the preparation (according to [17]), probably form fraction IV. This fact is proved by the accordance of UV absorption spectra recorded on each side of the peak with the literature data for these alkaloids (Table 1). We could say the same about homochelidonine and chelerytrine of the corresponding fractions V and VI.

The application of a considerable quantity of the preparation (up to 2 ml) on the column showed that the UV absorption spectrum of the sample taken on the 16$^{th}$ minute of the separation was identical to the berberine UV spectrum, and those of the samples at the 23$^{rd}$ minute (between peaks II and III, there was a barely perceptible peak) were identical to coptisine spectrum. With regard to this fact, we may suppose that there is only a little quantity of this alkaloid in *Ukrain* preparation. As it may be seen from the Table, the substances of fractions I and IV have similar absorption spectra. Along with protopine and allocryptopine, chelamine and chelamidine alkaloids have the same spectra. In [14] chelamine and chelamidine form the fraction of so-called *«methoxychelidonine»* that should be described in a more detailed way.

«Methoxychelidonine», as one of the specific minor alkaloids of the celandine roots, was discovered long time ago, but only in 1994 J. Slavik *et al*. determined that it is a mixture of three alkaloids: chelamine (67 %), chelamidine (5 %), and homochelidonine (28 %) [19]. High-performance chromatography (under conditions different from those used in [14]) showed that the first two alkaloids were poorly separated and came out practically as one peak, whereas homochelidonine has the retention time almost twice as long. This is in agreement with our data. The retention time is 16 min for Fraction I, and 32 min for homochelidonine. Thus, we may suppose that fraction I contains mainly chelamine, chelamidine, and some berberine.

As it was mentioned above, the substances of fractions II and III have similar absorbance maxima at 265, 283, and 314-324 nm (Fig. 7), and do not correspond to any of the known *C. majus* alkaloids. Therefore, it would be logical to suppose that substances of these fractions are the products of the alkaloids alkylation, when thiotepa molecule binds alkaloids of berberin group (absorbance maximum at 265 nm) and chelidonine group (absorbance maximum 283-288 nm). This fact can explain different retention times of substances with the same absorption spectra (in the form of two fractions), depending on the group of predominating alkaloids. But in that

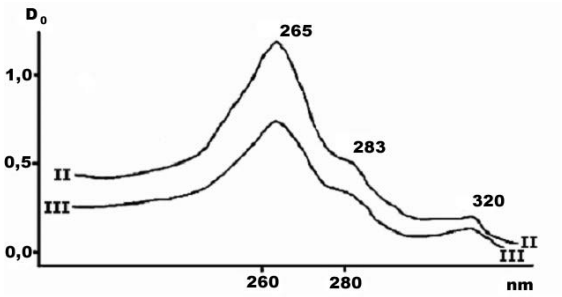


*Fig. 7. UV absorption spectra of fractions II and III from Fig. 6.*

case there is still an unexplained fact of sulfur and phosphorus absence in the final product. At the same time, taking into account the fact that hydrochloric acid treatment was used for the isolation of *Ukrain* from a reaction mixture, the hydrolysis of phosphoramide bonds of preparation is inevitable, and sulfur and phosphorus absence is fully explainable.

**Conclusions.** Thus, at present the composition of medical preparation *Ukrain* has not been established completely and its formula declared by its authors does not represent the real structure. Except for the major known *Chelidonium majus* alkaloids (berberine, coptisine, allocryptopine, protopine, chelidonine, homochelidonine, chelerythrine, sanguinarine, and probably minor alkaloids), *Ukrain* contains about 40 % of unidentified compounds.

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**Вивчення складу препарату «Україн» із використанням ВЕРХ та УФ-спектроскопії**

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**Резюме.** Методами ВЕРХ та УФ-спектроскопії досліджено склад протипухлинного препарату «Україн». Встановлено, що до нього, крім відомих алкалоїдів чистотілу великого (близько 10), входять сполуки з невстановленою структурою. За даними ВЕРХ, їх частка в препараті складає близько 40 %. Вони не є алкілованими алкалоїдами, бо сірки та фосфору в них не знайдено, а їх УФ-спектри не належать ні одному з відомих для чистотілу алкалоїдів. До складу лікарського препарату «Україн» не входять сполуки, які б відповідали заявленій для нього структурі, де на одну молекулу тіотефу припадає три молекули хелідоніну або будь-яких інших алкалоїдів чистотілу.

**Ключові слова:** *Україн*, чистотіл великий, алкалоїд, тіотеф, целандін.

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