Reference Standards Based on the Grosheimin and Cynaropicrin

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Article info

Received:
10 August 2015

Received and revised form:
18 October 2015

Accepted:
20 November 2015

Keywords:

Chartolepis intermedia Boiss., reference standards, grosheimin, cynaropicrin, chromatographic methods

Abstract

The aim of this work is the development of effective method for producing the reference standards of Grosheimin and Cynaropicrin for quality control of domestic herbal medicines, as well as project development concerning normative documents on reference standards of Grosheimin and Cynaropicrin, an introduction of reference standards to the Pharmacopoeia of Kazakhstan. This article discusses the method of allocation and purification of reference standards of Grosheimin and Cynaropicrin which are sesquiterpene lactones of guaiane type were obtained from the ethyl acetate extract of *Chartolepis intermedia* Boiss., using centrifugal distribution chromatography and high performance liquid chromatography, allows to obtain the qualitative target products. The effectiveness of developed technology has reduced the labor contribution in 2.0 times and as a consequence the prime cost of end products come down 3 times. Using of Grosheimin and Cynaropicrin as own reference standard of the Republic of Kazakhstan for monitoring of staged quality control of pharmaceutical production: access control, intermediate control and output control, it means the finished dosage form. There are some chromatographic analysis data of the samples. Such methods as IR and NMR- spectophotometries were applied for determination of molecular structure. Pharmacopoeial methods were used to determine the color, taste, odor and solubility of reference standards in various solvents. Projects for temporary Analytical Normative Documents on reference standards of Grosheimin and Cynaropicrin were developed in accordance with requirement of State Pharmacopeia of the Republic of Kazakhstan. On the basis of obtained results the database for State Pharmacopoeia of the Republic of Kazakhstan will be formed, as well as control over production of new pharmaceuticals from *Chartolepis intermedia* Boiss. and *Saussurea salsa* (Pall.) Spreng.

1. Introduction

Production of high-effective and safety medicines requires a comprehensive solution to problems at the stages of research the optimal synthesis methods of pharmacologically active substances, investigation of their physical and chemical properties, study of biological activity and toxicity, production setup and organization of finished-product output. All stages require analytical control realization using reliable methods for investigation and analysis.

To date, there is a shift of emphasis from finished product analysis to analytical process monitoring, it means quality-based products and should be controlled during production. This implies that the analytical process monitoring of medicines should be provided in full concordance with active normative documentation: technical specifications (TS), State Standards for raw and other materials, section of standard operating procedures «Production control», enterprise standards for intermediate goods, company instructions, Pharmaceutical Norms and Regulations and others.

It should be noted that in recent years there has been positive movement towards advanced use of physical and physical-chemical analysis methods. In particular, the spectral methods such as infra-red spectroscopy (IR) and ultraviolet spectophotometery, nuclear magnetic resonance spectroscopy and

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others are widely used here. Also, such chromatographic methods as high-performance liquid chromatography, gas-liquid chromatography and thin layer chromatography are actively used. Certainly, this is not about full elimination of chemical analysis of medicines and substances. However, the review of foreign pharmacopoeial monographs leads to the conclusion that leading codices are mainly used the physical and physicochemical methods in order to establish the authenticity, purity analysis and quantitative measurement physical and physicochemical methods.

Different non-chemical properties of pharmaceutical substances are estimated when using such methods and could be described theoretically one time and compare the experimental result with available data. However, the experimental conditions such as (temperature, humidity, and pressure) and equipment in every quality control center are independent. Also, the analyses are carried out by different people. In this regard, the chromatogram or spectrum that obtained for the same substance in the same laboratory and by the same person, but at different days, will vary. Accordingly, it is difficult to draw a conclusion about quality of a medicine on the basis of such variable data.

The way out of this situation was found long time ago. When using physical, chemical and biological analysis methods it is necessary to apply the reference standards. They represent characterized substances that subjected to the same analysis as test product during quality control. For example, the same position of the peaks in HPLC and GC chromatograms, or the same position of the spots in TLC chromatograms as well as coincidence of standard spectra and tested substances indicates the authenticity of the latest.

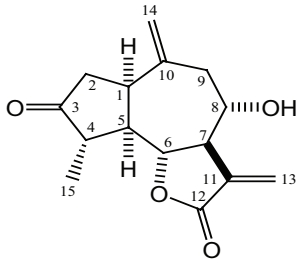
In world practice, the pharmacopoeial reference standards and universal reference standards of active pharmaceutical substances and their impurities are used for standardization of raw materials, pharmaceutical ingredients and finished dosage form. It includes the reference standards of vegetable Grade as well as physical and chemical material standards for calibration of measuring equipment. Reference standards are the factors, directly influencing on the quality of obtained laboratory results, allows to monitor the quality of all stages of pharmaceuticals production according to GMP standards. The European Pharmacopoeia includes 75 reference standards, the American Herbal Pharmacopoeia includes 36 reference standards, and the Russian State Pharmacopoeia includes more than 30 herbal reference standards [1].

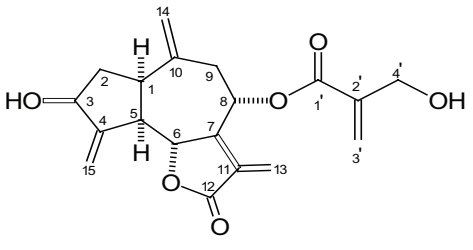
The above-mentioned reference standards were not included in the State Pharmacopoeia of the Republic of Kazakhstan [2]. In order to obtain the reference standards and specification documents for objective estimation of qualitative domestic pharmaceuticals the works on standardization of terpenoids, flavonoids, alkaloids and ecdysteroids are carried out in International scientific and production holding company “Phytochemistry”. Advanced pharmaceutical drugs on the basis of Grosheimin and Cynaropicrin which are sesquiterpene lactones are being developed in International scientific and production holding company “Phytochemistry”. Therefore, for quality control, production, and certification of crude drugs it necessary to develop the reference standards for Grosheimin (1) – where “Hartinol” is the active substance and Cynaropicrin (2) – where “Sausalin” is the active substance of the antiparasitic drug.

Grosheimin (1) is a sesquiterpene lactone of guaiane type has high antitumour, antiviral, antiphlogistic, and bactericidal activity [3–5]. The “Hartinol” is a pharmaceutical water-soluble form of Grosheimin, is obtained by chemical modification. Conducted researches of water-soluble form of Grosheimin have shown its prospectivity as advanced virus inactivating agent against influenza infection and it may be recommended as a means of expanded preclinical and clinical tests [6].

Cynaropicrin (2) is a sesquiterpene lactone of guaiane type possessing high antitumor, antibacterial, anti-protist and anti-trichomonas activities [5, 7] It is active component of the “Sausalin” antiparasitic pharmaceutical effect.

(1)





(2)

2. Experimental

The materials and methods are used for scientific researchers satisfy the requirements of State Pharmacopeia of the Republic of Kazakhstan, European Pharmacopoeia, United States Pharmacopeia, British Pharmacopeia, Pharmacopeia articles (PA), temporary pharmacopoeia articles (TPA) and other regulations are in force within the territory of the Republic of Kazakhstan.

2.1. Chemicals and reagents

Raw materials are aboveground part (leaves, flowers and bud) of the *Chartolepis intermedia* Boiss., (temporary analytical normative document TAND 42-483-12) were gathered in September 2013 in the neighborhood of Akbastau river of Abay district, Karaganda region of the Republic of Kazakhstan, were dried for 2 weeks at room temperature. The herbarial sample was added to the database of herbarial fund of JSC International Research and Production holding company "Phytochemistry" Raw materials are aboveground part (anthodium) of *Saussurea salsa* (Pall.) Spreng. *herba* (AND 42-6056-15).

The extract is an ethyl acetate extract of *Chartolepis intermedia* Boiss.

Substances are grosheimin (project of AND), "Hartinol" (project of AND), and dry extract of *Saussurea salsa* (Pall.) Spreng. (AND 42-6173-15).

The finished dosage forms are "Hartinol" lyophilized powder (project of AND) and "Sausalin" tablets (project of AND).

Hexane (Hex), ethyl acetate (EtOAc), acetonitrile (CH_3CN) (CJSC "Kupavnareaktiv", Russia), ethanol (C_2H_5OH), methyl alcohol (MeOH) (Merck, Germany) were used for extraction and chromatography are divided into following grades: "analytical grade", "chemically pure", and "reagent grade", distilled water (H_2O).

2.2. Equipment

Extract separation was carried out using FCPC system, equipped with 200 ml of rotor which was produced by "Kromaton Technologies firm" (Angers, France). The total volume of rotor is 200 ml. The rotational speed was subjected from 0 to 2000 revolutions per minute. The FCPC system was equipped with an isocratic pump "Alpha" (Kromaton, Angers, France) with a flow rate from 0 to 100 ml/min. The detection was carried out under ultraviolet light at the wavelength of 204 nm.

Preparative separation was carried out using HPLC chromatograph (Varian, USA), which consist of the following items: the pump "SD-1", column of 250×9.4 mm is filled with sorbent "Microsorb 60-8 C18" (10 micron), UV detector "ProStar 325", Rheodyne loops with volume of 10 μL, and fractional collector "ProStar 701".

HPLC analyses were performed using Chromatograph "Agilent 1100" (Agilent Technology, USA), which consist of the following items: the pump "QuatPump G1311A", UV detector "G1314A", Rheodyne loop with volume of 20 μL, degasifier "G1322A" with volume of 4.6×150 mm is filled with "Zorbax SB-C18" with particle size of 5 micron at room temperature. NMR spectra was recorded on a Bruker DRX-500 spectrometer (operating frequency is 500.13 MHz for ^{1}H NMR and 125.76 MHz for ^{13}C NMR, δ-scale) using standard software of the Bruker company to register two-dimensional spectra.

For performance of physical and chemical investigations the following professional equipment such as Infrared spectrometer "Termo Nicolet Avatar-360" (USA), UV spectrophotometer "Helios-β" (Great Britain), an Eurovector 3000 for elemental analyzer (Italy), melting point tester «Boetius» (Germaty) was used.

3. Results and Discussion

Previously, for the development of Grosheimin and Cynaropicrin the multiple methods as well as expensive, flammable, toxic organic solvents such as (chloroform, benzene, petroleum-ether, acetone, etc.) were used, but the development of Cynaropicrin was not performed [3–5, 8].

Therefore, for the development of Grosheimin and Cynaropicrin from *Chartolepis intermedia* Boiss there were used advanced chromatographic methods such as centrifugal distribution chromatography and high-performance liquid chromatography for isolation and purification of active compounds.

For the first time centrifugal partition chromatography was used for isolation and purification of Grosheimin and Cynaropicrin from the ethyl acetate extract of *Chartolepis intermedia* Boiss. Separation from the ethyl acetate extract of *Chartolepis intermedia* Boiss was carried out with the help of FCPC-A200 device. The rotor volume was 250 ml. Solvent system has included: hexane, ethyl acetate, acetonitrile and the water (1: 1: 1: 1). 2.3 g for the sample, 15 ml was upper (stationary) phase and 10 ml was mobile phase. 25 ml of sample was introduced into the chromatograph. UV

detection was carried out at 220, 254, 360, and 460 nm.

Rotational speed was 1400 rev/min. The method of separation was the double one (extrusion and elution).

- The upward elution was used. Mobile phase was the upper layer (non-polar). The eluent flow rate was 2.5 ml/min and fractions were collected in 1 min. 62 Fractions were collected.

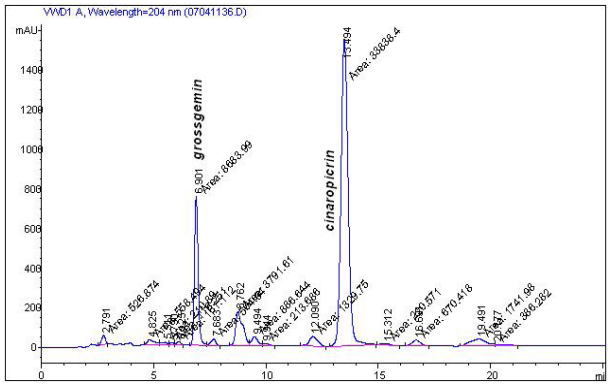
- The upward extrusion was used. Mobile phase was the lower layer (polar). The eluent flow rate of was 2.5 ml/min and fractions were collected in 1 min. 17 Fractions were obtained.

According to the HPLC results it was found that grosheimin and cynaropicrin were absent in fractions F_1-F_{19}. The content of grosheimin in fractions F_{20}-F_{30} was from 6.25% to 22.23%. The content of cynaropicrin was from 62.85% to 78.21%. Grosheimin and cynaropicrin were present in fractions F_{31}-F_{33} in trace amounts. Figure 1 shows chromatograms of the analysis of fractions F_{20}, F_{22}, F_{24}, F_{30}.

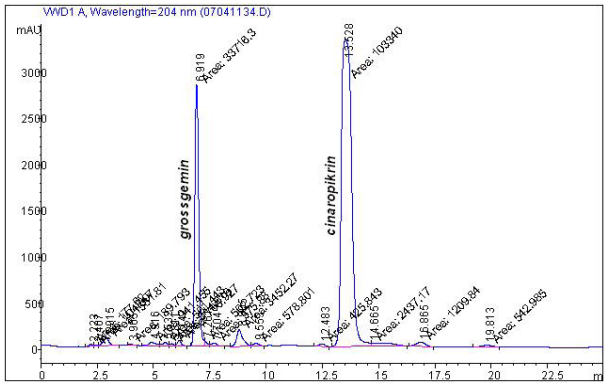
Fractions F_{20}-F_{30} were combined by analysis. The combined obtained fractions contains 17% of grosheimin and 72% of cynaropicrin.

Since the separation of the ethyl acetate extract of *Chartolepis intermedia* Boiss. with the help of centrifugal partition chromatography failed to isolate grosheimin and cynaropicrin as individual compounds high-performance liquid chromatography was used for further purification of the target products.

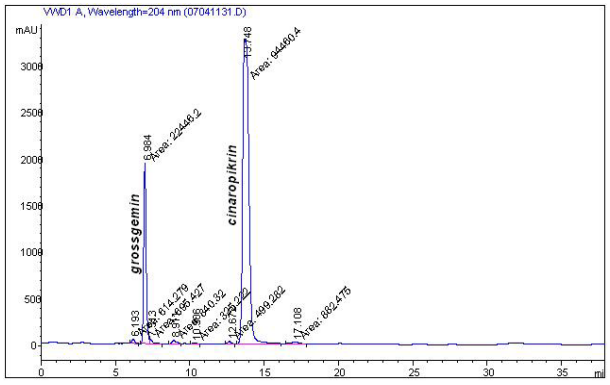
Separation of the combined fraction containing grosheimin and cynaropicrin was carried out on a preparative HPLC set-up using a reversed-phase HPLC with the help of a preparative 9.1×250 mm column packed with the Microsorb 60-8 C18 sorbent with particle size 10 microns. Mobile phase was methanol-water (50:50). When the volumetric flow rate was 4 ml per minute complete separation of the sample had taken place within 30 minutes. The column temperature was room temperature. UV detection was carried out at 204 nm. Fraction collection was carried out using a fractional collector according to the separation chromatogram obtained.



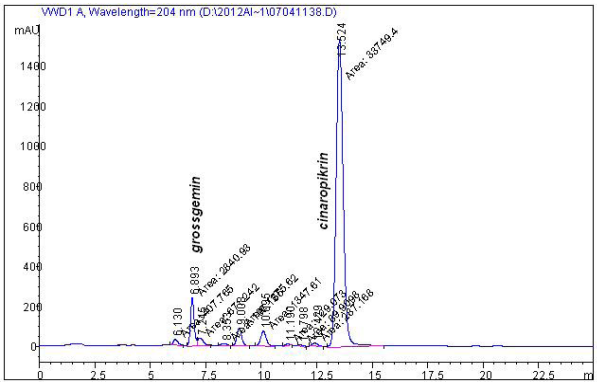
The chromatogram of fraction F_{20} that contains 16.13% of grosheimin and 62.85% of cynaropicrin.



The chromatogram of fraction F_{22} that contains 22.2% of grosheimin and 68.34% of cynaropicrin.

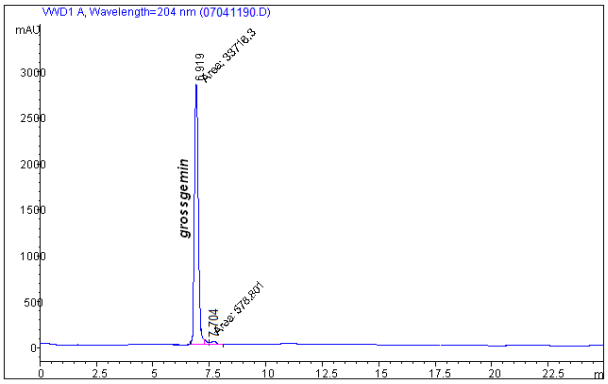


The chromatogram of fraction F_{24} that contains 18.59% of grosheimin and 78.21% of cynaropicrin.

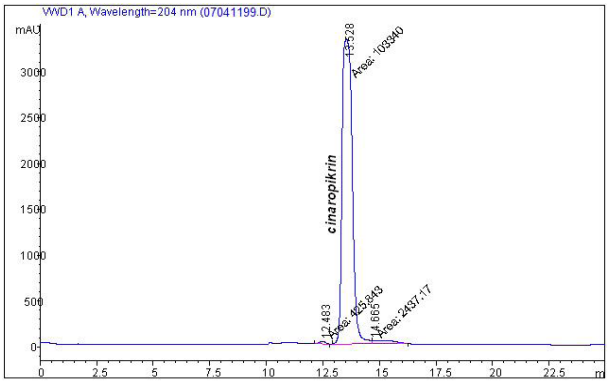


The chromatogram of fraction F_{30} that contains 6.25% of grosheimin and 77.34% of cynaropicrin.

Fig. 1. The HPLC chromatogram of fractions obtained at separation of *Chartolepis intermedia* Boiss. with the help of centrifugal partition chromatography using a 15×0.46 cm Zorbax SB-C18 column (5 μm). The mobile phase was methanol:water (50:50 v/v). Flow rate was 0.5 mL/min. Detection was performed with the help of a UV detector at 204 nm.



The chromatogram of grosheimin with purity 99.39 %.



The chromatogram of cynaropicrin with purity 98.74%.

Fig. 2. The HPLC chromatograms of grosheimin and cynaropicrin obtained after separation of the combined fraction with the help of preparative chromatography using a 15×0.46 cm Zorbax SB-C18 column (5 μm). The mobile phase was methanol:water (50:50 v/v). Flow rate was 0.5 mL/min. Detection was performed with the help of a UV detector at 204 nm.

In the result of separation there were obtained fractions that contain the target products. After evaporation of the solvent grosheimin with purity of 99.39% and cynaropicrin with purity of 98.74% were obtained (Fig. 2). The yield of grosheimin was 6.5% (0.14%) and the yield of cynaropicrin was 24.7% recalculated on the total extract (recalculated on the weight of air-dried raw materials).

Grosheimin (1) is a white crystalline powder with yellowish tinge, odourless, the melting point is 200–203 °C (ethanol) is soluble in 96% of ethanol, highly soluble in ethyl acetate, chloroform, practically insoluble in the water $[\alpha]_D^{20}+115^0$ (c 1.0; ethanol). The Grosheimin in IR spectrum has some intensive absorption bands are typical for such groups as 3474 cm^{-1} (OH group), 1741 cm^{-1} (C=O γ-lactone), 1648 cm^{-1} (C=C), 1399, 1167 cm^{-1} (exocyclic methylene group is coupled with the carbonyl of γ-lactone). The Grosheimin in UV spectrum has the maximum absorption at the wavelength of 201±2 nm, is typical for the exocyclic methylene, being in conjugation with the carbonyl. The elemental analysis: it was found %: C 68.80; H, 6.89; it was calculated %: C, 68.70; H 6.87. In molecule of Grosheimin there are 6 asymmetric carbons are located at C-1, C-4, C-5, C-6, C-7, C-8.

The Grosheimin in mass spectrum (1) has a peak of molecular ion with small intensity of m/z 262 [M^+], which is equivalent of its molecular weight. Further, the ion decomposition is occurred in two directions. Ion with m/z 244 is formed because of elimination of water molecule, which indicates the presence of hydroxyl group in initial guayanolide. The loss of CO group by this fragment leads to ion formation with m/z 216 type. The fragmentation of molecular ion by the second path begins with emission of CO group (peak with m/z 234) and subsequent loss of water molecule (peak with m/z 216 type). Ion with m/z 200 is formed because of elimination of water molecules, which confirms the presence of keto group in grosheimin molecule. In NMR ^{13}C spectrum of Grosheimin there are 4 singlet, 6 doublet, 4 triplet and 1 quartet signals are typical for 6 sp^2- and 9 sp^3- hybrid carbon atoms.

The following signals are observed in 1H NMR spectrum of Grosheimin molecule: methyl proton at C-4, resonate as a doublet at 1.25 ppm. Proton H-5, resonates as a multiplet at 2.45, The exomethylene methyl group at C-13 gives rise to two symmetrical doublet of doublets centered at 6.29 ppm with splittings 3 Hz and 4 Hz and 6.36 ppm with splittings 3 Hz and 4 Hz, respectively. A multiplet of methine proton H-7 is centered at 3.15 ppm and a triplet of lactone proton H-6 resonates at 4.08 ppm (J=9.0 Hz). The nature of the signal splitting of the lactone proton indicates that the lactone ring is located at C6-C7 of the basic skeleton, and the spin-spin coupling constant indicates a trans-coupling. There are also other signals in the ^1H NMR spectrum of the Grosheimin molecule. Proton H-1 resonates as a multiplet at 3.25 ppm. A doublet of doublets with splittings 4 Hz and 6 Hz resonates at 2.53 ppm. A doublet with a coupling constant of 9 Hz at 2.64 ppm corresponds to protons H-2a and H-2b. A signal of proton H-4 resonates as a doublet at 2.59 ppm with a coupling constant of 9 Hz. Multiplets of protons H-8 and H-9a resonate at 3.82 ppm and 2.28 ppm, respectively. Proton H-9b gives rise to a signal at 2.37 ppm, and signals of singlets at 4.8 ppm and 5.1 ppm belong to the exomethylene methyl group at C-14.

On the basis of executed work there were formed documentation packages for reference sample of Grosheimin and reference sample of Cynaropicrin, which had been submitted to the National Center for Expertise of drugs.

The Grosheimin (1) is necessary as domestic reference standard of the Republic of Kazakhstan for monitoring of staged quality control of pharmaceutical production: access control – it means quality control of raw materials *Chartolepis intermedia* Boiss., intermediate control – it meant quality control of Grosheimin substance, output control – it means the finished dosage form of antiviral drug "Hartinol".

The identification on external and microscopic features as well as qualitative estimation of active substance in raw material is the basic stage for standardization of raw materials [9].

The *Chartolepis intermedia* Boiss is an experimental raw material is characterized by the following external features: the stem is upright, thin-ribbed; the leaves are pediculate, elliptically-back- lanceolar; the leaf venation is pinnate; the flowers are yellow; the feathery is tuft; the bracts are multiserial.

3.1. Identification

When considering the microscope slide from above it can be seen the epidermal cells are covered with thick bed of cuticle; the mesophyll consists of several lines of palisade cells, are located on either side of spongy parenchyma, it means that has isolateral-palisade structure type; there are also multicellular trichomes. The cross section of the leaf shows the structure of conducting bundle, consisting of phloem and xylem and is related to collateral structure type.

Identification of raw materials was confirmed by the presence of terpenoids and lactones. TLC test was performed using a silica gel plate of a "Sobfill" type. The mobile phase was petroleum ether: ethyl acetate (1:1). The chromatogram showed a spot of a test solution at the same level as a spot, which corresponds to a grosheimin reference standard. Indicators such as impurities, weight loss on drying, total ash, acid-insoluble ash were determined in accordance with the State Pharmacopeia of the Republic of Kazakhstan I, Vol. 1, "Test methods for medicinal plant raw materials", "Technique of microscopic and microchemical investigation of medicinal plant raw materials". When considering the specimen in the microscope there are corresponding diagnostic features are observed.

The content of impurities. Not more than 2%.

Weight loss on drying. Not more than 13.0%.

Total ash. Not more than 12.0%.

The ash is insoluble in muriatic acid. Not more than 3.5%.

3.2. Quantitative determination

At the present time the chromatographic methods are most common advanced analysis methods for herbal raw materials. The most important feature of these methods is the objective assessment of quantitative content of pharmacologically active substances, which in turn determines the quality of raw material. The quantitative determination of biologically active substances in raw material such as *Chartolepis intermedia* Boiss is suggested to perform using high performance liquid chromatography. It is characterized by the accuracy, repeatability, and linear dependence in analytical field of ± 30% of the declared in the AND. It helps to use it for a reliable assessment of the quantitative content of Grosheimin in *Chartolepis intermedia* Boiss.

The content of Grosheimin in dry raw material should be at least 0.05% (Fig. 3).

3.3. Substance control

The Grosheimin substance is a white crystalline powder with yellowish tinge, odorless, is soluble in ethanol 96%, freely soluble in ethyl acetate, chloroform, practically insoluble in purified water. For determination of the authenticity it was suggested some methods such as IR spectroscopy and ultraviolet spectrophotometry, qualitative reaction for the presence of terpenoids and melting point determination.

The purity of Grosheimin substance was determined by high performance liquid chromatography method (HPLC), using "Hewlett Packard Agilent 1100 Series" apparatus in isocratic mode. The substance content is not less than 98%. (Fig. 3).

3.4. Output control, finished dosage form

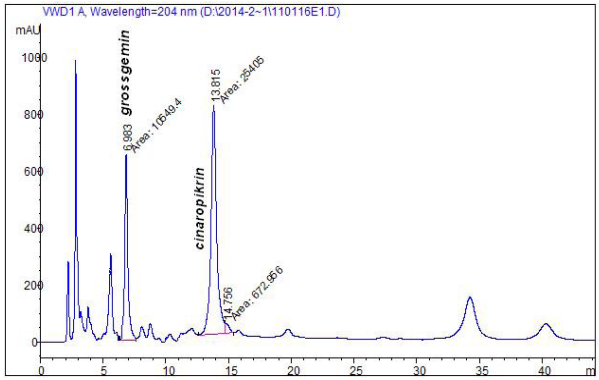
The antiviral drug "Hartinol" is a powder varying from cream-coloured to light brown color, odorless. The "Hartinol" is a lyophilized powder without auxiliary substances. The content of basal substance is not less than 97%. It is soluble in ethanol 96%, freely soluble in purified water, practically insoluble in diethyl ester, and chloroform. For determination of the authenticity it was suggested some methods such as IR spectroscopy and

ultraviolet spectrophotometry, qualitative analysis for the presence of terpenoids and iodides. For determination of quantitative content of active substance the High performance liquid chromatography has been suggested. The qualitative test for the presence of terpenoids and iodides was also used.

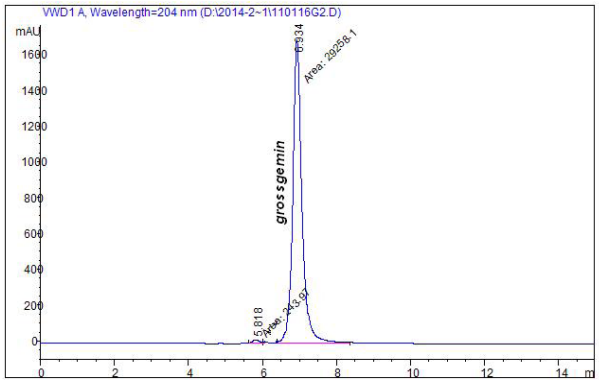
The technique of determination of the quantity of the active component and the main related impurity that is grosheimin in the substance and the "Hartinol" finished dosage form by HPLC was developed. According to validated characteristics the developed method is specific for the determination of an active component and grosheimin in the substance and the finished dosage form. It is characterized by accuracy, repeatability, and linear dependence in the analytical field of ± 30% of the declared in the AND. It helps to use it for a reliable assessment of the quantitative content of grosheimin in the substance and the "Hartinol" finished dosage form (Fig. 4).

The content of the active substance and grosheimin in the substance and the "Hartinol" finished dosage form was not less than 97.0% and not more than 3.0%, respectively.

Cynaropicrin (2) has dark yellow color with a greenish tinge, odorless, possessing $C_{19}H_{22}O_6$, it has $[\alpha]_D^{20}$+108.6^0 (c 1.19; ethanol). [10]. The Cynaropicrin is soluble in ethanol 96%, slightly soluble in ethyl acetate, chloroform, and insoluble in the water

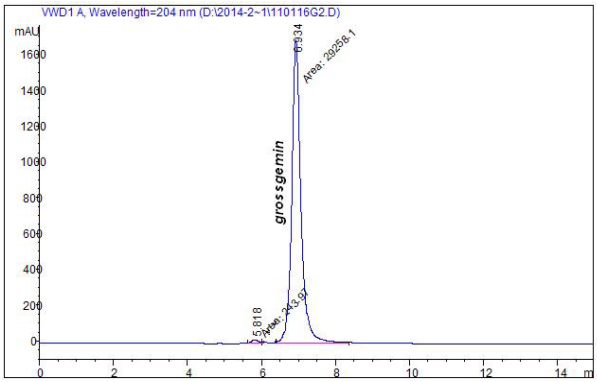


The chromatogram of the quantitative determination of grosheimin in the *Chartolepis intermedia* Boiss. raw material.

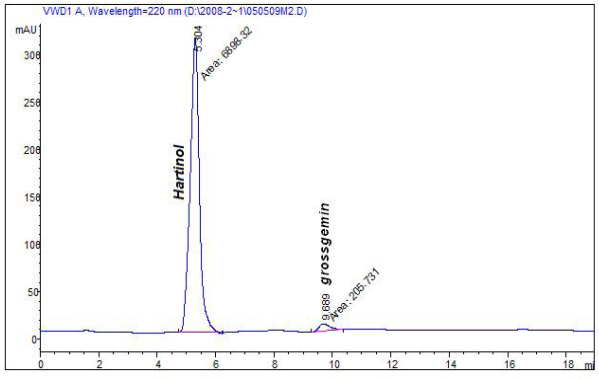


The chromatogram of the quantitative determination of grosheimin in the grosheimin substance.

Fig. 3. The HPLC chromatograms of quantitative determination of grosheimin in *Chartolepis intermedia* Boiss. and in the grosheimin substance. The analysis was carried out with the help of a 15×0.46 cm Zorbax SB-C18 column (5 µm). The mobile phase was methanol:water (50:50 v/v). Flow rate was 0.5 mL/min. Detection was performed with the help of a UV detector at 204 nm.



The chromatogram of quantitative determination of an active substance (98.2%) and grosheimin (1.8%) in the "Hartinol" substance.



The chromatogram of quantitative determination of an active substance (98.5%) and grosheimin (1.5%) in the "Hartinol" finished dosage form.

Fig. 4. The HPLC chromatograms of quantitative determination of an active component and grosheimin in the substance and the "Hartinol" finished dosage form. The analysis was carried out with the help of a 15×0.46 cm Zorbax SB-C18 column (5 µm). The mobile phase was methanol:acetonitrile (50:50 v/v). Flow rate was 0.2 mL/min. Detection was performed with the help of a UV detector at 220 nm.

Cynaropicrin in IR spectrum (2) has some intensive absorption bands are typical for such groups as 3428 cm^{-1} (OH-group), 2931, 2872, 1756 cm^{-1} (C=O γ-lactone),, 1715 cm^{-1} (methacrylic ester), (C=C), 1660 cm^{-1} (C=C) 1376 cm^{-1}, 1270 cm^{-1} (exocyclic methylene group is coupled with the carbonyl of γ-lactone). The Cynaropicrin in UV spectrum has the absorption maximum at the wavelength of 204±2 nm, is typical for the exocyclic methylene, being in conjugation with the carbonyl. In molecule of Cynaropicrin there are asymmetric carbons are located at C-1, C-3, C-5, C-6, C-7, C-8.

In mass spectrum of Cynaropicrin (2) there is no a molecular ion peak, but the fragment ions are registered in small amount, and this fact makes the molecular structure determination process difficult, because the Cynaropicrin is oil-like substance and at the temperature above 80 °C its molecule is exposed to thermal decomposition. In NMR ^{13}C spectrum of Cynaropicrin there are 6 singlet 6 doublet, 7 triplet signals.

The following signals are observed in NMR spectrum of Cynaropicrin molecule: singlets at 6.31 and 5.93 ppm that correspond to protons H-3a and H-3b of the exomethylene double bond of the 4'-hydroxymethacrylic acid residue, respectively. Signals of two protons at C-4 resonate as a broadened doublet with a coupling constant of 4.0 Hz at 4.96 ppm and a multiplet of proton 4'-OH of the 4'-hydroxymethacrylic acid residue resonates at 2.20 ppm. Doublets with a coupling constant of 3 Hz at 6.18 and 5.61 ppm are characteristic for the protons H-13a and H-13b of exomethylene group of a lactone ring, respectively. Singlets of protons H-14a and H-14b resonate at 5.11 and 4.82 ppm, respectively, and doublets of protons H-15a and H-15b resonate at 5.27 and 4.85 ppm with a coupling constant of 12.0 Hz, respectively. A multiplet at 5.13 ppm indicates the presence of the H-8 proton, which is geminally located to the ester group, and doublet of doublets with coupling constants of 9.0 Hz and 10.0 Hz at 4.79 ppm corresponds to the proton H-6 of a lactone ring. A multiplet of a methine proton H-7 is centered at 3.13 ppm.

The principal of splitting of lactone proton signal indicates that the lactone ring of Cynaropicrin is located at C6-C7 of the basic skeleton, but the spin-spin interaction constant indicates on its trans-connection. Also there are presented the proton signals H-1, multiplet at 3.49 ppm., multiplets at, 2.45 ppm and 2.64 ppm are corresponding to protons H-2a and H-2b, proton signals H-3 in the form of doublet at 3.80 ppm with Spin-spin coupling constant of J= 6.0 Hz, triplet of protons H-5 with Spin-spin coupling constant of J=10.0 Hz at 2.30 ppm, of doublet of protons H-9a with Spin-spin coupling constant of J_1=4.0, J_2=14.0 Hz and H-9b with Spin-spin coupling constant of J_1=2.0, J_2=14.0 Hz at 2.70 and 2.39 ppm correspondently and multiplet of protone is 3-OH at 3.49 ppm.

Storage requirements for Cynaropicrin: In a dry, dark place at the temperature of 2 °C.

On the basis of executed work there were formed documentation packages for reference sample of Cynaropicrin and reference sample of Cynaropicrin, which had been submitted to the National Center for Expertise of drugs.

The Cynaropicrin (2) is necessary as domestic reference standard of the Republic of Kazakhstan for monitoring of production quality control of novel antiparasitic herbal medicinal product "Sausalin" on the basis of dry extract from *Saussurea salsa* (Pall.) Spreng., the active pharmaceutical ingredient is *Saussurea salsa* (Pall.) Spreng. – tablets of herbal medicinal product "Sausalin" [11].

Quality control of the raw material *Saussurea salsa* (Pall.) Spreng. was carried out in accordance with Analytical Normative Documents RK 42-6056-15 with following parameters: external features, identification, weight loss on drying, total ash, the ash which is insoluble in muriatic acid and quantitative determination.

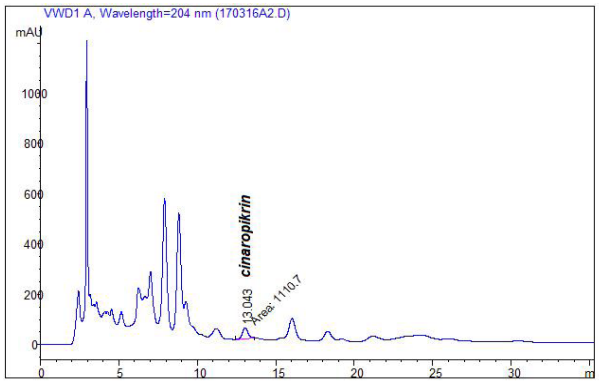
3.5. Total Raw material

Stems are furrowed. Leaves are glabrous, more or less scabrous because of short hard fibers, from below are dotted with many point glands; lowers are petiolate, lyrated- pinnatifid. Anthodes are numerous, with thick corymbs. The flowers are narrowly tubular, flower-crown is tricuspidate.

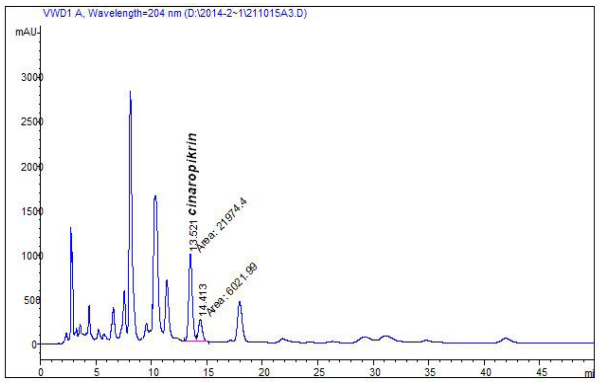
3.6. Gritted raw material

The pieces of stems, anthodes, flowers, leaves are passing through a basket with holes with diameter of 8 mm. The color is green with violet-pink inclusions with white coronules. The smell is bitter-grassy and the taste is bitter.

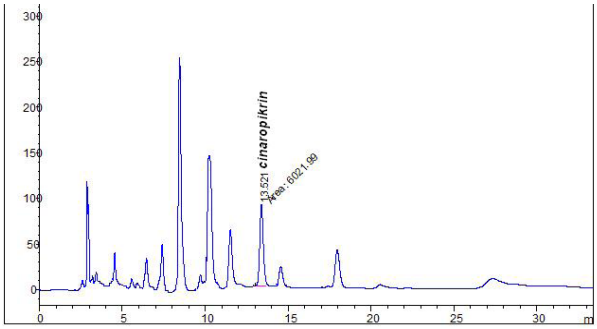
Identification of raw materials was carried out in accordance with the State Pharmacopeia of the Republic of Kazakhstan I, Vol. 1, "Test methods for medicinal plant raw materials", "Technique of microscopic and microchemical investigation of medicinal plant raw materials". When considering the specimen in the microscope there are corresponding diagnostic features are observed.



The chromatogram of quantitative determination of cynaropicrin in the *Saussurea salsa* (Pall.) Spreng. raw material.



The chromatogram of quantitative determination of cynaropicrin in the *Saussurea salsa* (Pall.) Spreng. dry extract.



The chromatogram of quantitative determination of cynaropicrin in the "Sausalin" tablets.

Fig. 5. The HPLC chromatograms of quantitative determination of cynaropicrin in the *Saussurea salsa* (Pall.) Spreng. raw material, the *Saussurea salsa* (Pall.) Spreng. dry extract and "Sausalin" tablets. The analysis was carried out with the help of a 15×0.46 cm Zorbax SB-C18 column (5 µm). The mobile phase was methanol:water (50:50 v/v). Flow rate was 0.5 mL/min. Detection was performed with the help of a UV detector at 204 nm.

The content of impurities is not more than 2%.

Weight loss on drying. Not more than 13.0%.

Total ash. Not more than 12.0%.

The ash is insoluble in muriatic acid. Not more than 3.5%.

Quantitative determination. Determination is carried out by liquid chromatography method.

The content of Cynaropicrin in dry raw material must be at least 0.1% (Fig. 5).

Quality control of the substance was performed according to Analytical Normative Documents RK 42-6173-15.

Dry extract *Saussurea salsa* (Pall.) Spreng. is an amorphous powder of brown color with greenish tinge, with a specific odor, and bitter taste. It's hygroscopic. It's freely soluble in chloroform and ethanol, practically insoluble in the water.

The authenticity was determined by thin layer chromatography and high-performance liquid chromatography.

The quantitative determination of sesquiterpene lactones was determined by high-performance liquid chromatography.

The content of sesquiterpene lactones in the substance, in equivalent to Cynaropicrin (dry raw material) should not be less than 1.0% (Fig. 5).

Quality control of finished dosage form - tablets of herbal medicinal product "Sausalin" was carried out in accordance with AND RK 42-6173-15.

"Sausalin" is the tablet with inclusions, has plain and double radius surface, engravure, bevelled edge, the color varying from light brown to greenish.

Quality control over the tablets was carried out upon indications: average tablet weight, disintegration, dissolution, friability in accordance with the State Pharmacopeia of the Republic of Kazakhstan Vol. 1.

The quantitative determination of Cynaropicrin in "Sausalin" tablets was determined by high-performance liquid chromatography.

The content of Cynaropicrin in medicinal product should not be less than 0.25% assuming average weight of one tablet (Fig. 5).

Considering the fact that reference standards are necessary for quality control of medicines production, the aim of this work is the development of effective method for producing the reference standards of Grosheimin and Cynaropicrin for quality control of domestic herbal medicines, as well as project development concerning normative documents on reference standards of Grosheimin and Cynaropicrin, an introduction of reference standards to the Pharmacopoeia of Kazakhstan.

It was established that the best methods for separation and purification of pharmacologically active substances (Grosheimin and Cynaropicrin) from ethyl acetate extract of *Chartolepis intermedia* Boiss., providing a quantitative yield of the qualitative target products using centrifugal distribution chromatography and high performance liquid chromatography. The effectiveness of developed technology has reduced the labor contribution in 2.0 times and as a consequence the prime cost of target products come down 3 times. The developed technology has variety of advantages: advanced production, competitive automatization, decrease of process time, absence of toxis solvents.

The method for the quantitative determination of grosheimin and cynaropicrin in plant raw materials such as *Saussurea salsa* (Pall.) Spreng. and *Chartolepis intermedia* Boiss., "Hartinol" and "Sausalin" substances and pharmaceuticals by HPLC was developed. Its validity was proved. According to the results of investigation there were elaborated the quality specifications for the reference standard of grosheimin and the reference standard of cynaropicrin including description, solubility, identification (IR spectrum, UV spectrum, qualitative reaction for terpenoids), melting point (for grosheimin), water and related impurities (HPLC), quantitative determination (HPLC), marking, transportation, storage, and shelf life. Based on the analysis results of 5 series of pilot batches projects of the analytical regulatory documents for reference standards of grosheimin and cynaropicrin were elaborated in accordance with the requirements of the State Pharmacopeia of the Republic of Kazakhstan.

There were formed packages of documents that contain originals of materials, which prove clearly the authenticity of grosheimin and cynaropicrin. Packages of documents for the reference standard of grosheimin and the reference standard cynaropicrin were submitted to the National Center for Expertise of pharmaceuticals.

As a result of the work carried out for the first time grosheimin and cynaropicrin are included into the State Pharmacopoeia of the Republic of Kazakhstan (Vol III, Section 5.12, p. 122) as reference standards, which are designed to identify and quantify the active components in medicinal plant raw materials and medicinal plant preparations.

4. Conclusion

As a result of this work a new method for separation and purification of pharmacologically active substances (Grosheimin and Cynaropicrin) from ethyl acetate extract of *Chartolepis intermedia* Boiss., using centrifugal distribution chromatography and high performance liquid chromatography have been developed. The effectiveness of developed technology has reduced the labor contribution in 2,0 times and as a consequence the prime cost of target products come down 3 times. The developed technology has variety of advantages: advanced production, competitive automatization, decrease of process time, absence of toxis solvents.

The temporary Analytical Normative Documents for reference standards of Grosheimin and Cynaropicrin were developed in accordance with requirements of State Pharmacopoeia of the Republic of Kazakhstan.

As a result of this work the Grosheimin and Cynaropicrin were included to State Pharmacopoeia of the Republic of Kazakhstan for the first time (Vol III, Section 5.12, p. 122) as reference standards are designed for identification and quantitative determination of biologically active substances in medicinal plant raw materials and medicinal plant preparations.

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