CRYSTALLINE GLYCYRRHIZIC ACID SYNTHESIZED FROM COMMERCIAL GLYCYRRAM. IMMUNOMODULANT PROPERTIES OF HIGH-PURITY GLYCYRRHIZIC ACID

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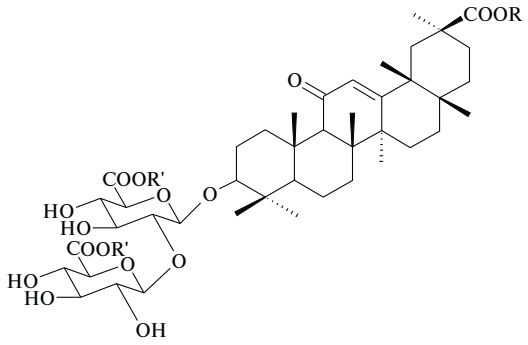
Glycyrrhizic acid (GA, I) is a triterpene glycoside representing the main active component of licorice root extract obtained from plants of the *Glycyrrhiza glabra L.*, *Glycyrrhiza uralensis Fisher*, or *Glucyrrhiza Korshinskyi Grig*. species. GA preparations exhibit various types of pharmacological activity, including antiviral [1, 2] and antitumor properties [3, 4]. Small doses of GA stimulate the production of γ-interferon both *in vitro* and *in vivo* [5, 6]. It was also reported that GA and its derivatives are capable of inhibiting HIV and Marburg viruses [7, 8].



I: R = R^1 = H, II: R = R^1 = K, III: R = K, R^1 = H.

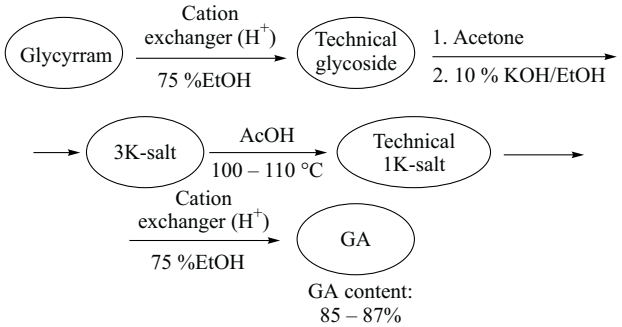
A convenient raw material for the synthesis of pure GA is offered by glycyrram – a monoammonium salt of glycyrrhizic acid, which is a commercially available antiinflammatory compound [9]. Also well developed are the schemes for isolating GA and glycyrram from a commercial dry licorice root extract, with the product purification stage employing domestic ion exchange resins [10, 11].

The purpose of this study was to develop a convenient method for obtaining high-purity (> 96%) crystalline GA from its commercial monoammonium salt (technical glycyrram). We employed a raw material representing technical glycyrram containing 75 ± 2% of the parent compound, available from the Chimkentbiofarm corporation (Chimkent, Kazakhstan).

Previously [12], we proposed a method for the synthesis of technical GA from commercial glycyrram. According to this technology, a glycyrram suspension in 75% aqueous ethanol is treated with cation exchangers (KU-2, KU-2-8, or KSP-4p) in the H^+ form taken in a 1 : 1 (w/w) ratio. The subsequent quantitative analysis by HPLC using a reversed-phase column showed that the obtained technical glycoside contains no more than 75 ± 2% of glycyrrhizic acid.

We propose two schemes for purification of the technical glycoside obtained from commercial glycyrram:

Scheme 1



These schemes include the following main stages:

(1) Synthesis of technical glycoside from commercial glycyrram with the aid of sulfoacid cation exchangers in the H^+ form in 70 – 75% ethanol;

(2) Dissolution of technical glycoside in acetone;

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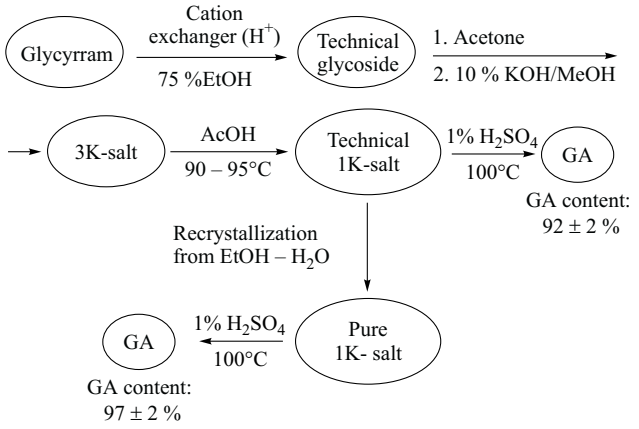
(3) deposition of tripotassium salt (3K-salt, II);

(4) conversion of 3K-salt into monopotassium salt (1K-salt, III) by crystallization from fivefold (scheme 1) or tenfold (scheme 2) volume of glacial acetic acid (AcOH);

(5) isolation of GA from 1K-salt.

Scheme 2



According to the first scheme, GA was isolated from technical 1K-salt using sulfoacid cation exchangers (KU-2, KU-2-8, KSP-4p, Dowex-50, or Amberlite IR-120) in the H^+ form. The target glycoside yield was 70 – 72% (calculated for 75% purity of the initial glycyrram). The GA content in the product did not exceed 87% (HPLC data). The conversion of 3K-salt into 1K-salt by crystallization from a fivefold volume of AcOH is accompanied by considerable losses of the target glycoside, because the salt solution preparation requires heating the mixture above 100°C. Prolonged heating of the 3K-salt in AcOH is accompanied by resinification of the substance or by the O-acetylation reaction at the alcohol groups of GA.

TABLE 1. The effect of Glycyrrhizic Acid on the Humoral Immunity Response and Delayed-Type (DT) Cell Immunity Reaction

Preparation	Dose, mg/kg	AFC number		DT response[a] × 10^{-3}	
		Per 10^6 splenocytes	Whole spleen	Negative control[b]	Positive control[c]
Control		222 ± 25	27,280 ± 227	0.25 ± 0.04	0.45 ± 0.04
GA	5	231 ± 21	25,265 ± 210 ($p < 0.05$)		0.52 ± 0.02 ($p < 0.05$)
	25	223 ± 19	20,750 ± 2010		0.54 ± 0.03 ($p < 0.05$)
	50	375 ± 38 ($p < 0.05$)	32,235 ± 2105 ($p < 0.05$)		0.49 ± 0.05
	200	184 ± 14	21,330 ± 2120		0.56 ± 0.06

Notes: [a] Each test group contained 16 mice, and control groups contained 60 mice; [b] the negative control group was not subjected to the sensitization treatment (only a challenging antigen dose); [c] the positive control group received both the model sensitization treatment and the challenging antigen dose.

In order to increase the yield of 1K-salt, we doubled (from five- to tenfold) the amount of AcOH used to convert 3K-salt into 1K- salt This allowed the crystallization process to be carried out at a lower temperature (90 – 95°C in scheme 2) and increased the 1K-salt yield up to 85 – 90% of the theoretical level. In order to increase the final product purity, we developed a new method for the synthesis of GA from glycyrram and succeeded in obtaining the target glycoside containing 97 ± 2% of the parent compound.

According to this refined scheme, commercial glycyrram containing 75 ± 2% of GA (HPLC data) was treated with sulfoacid cation exchangers (KU-2, KU-2-8, KSP-4p, Dowex-50, or Amberlite IR-120 in the H^+ form) taken in a 1 : 1 (w/w) ratio. The process was conducted in 70 – 75% aqueous ethanol at room temperature. The obtained technical glycoside was dissolved in acetone and converted first to the 3K-salt (by adding a 10% alcohol solution of KOH) and then to the 1K-salt by crystallization from a tenfold excess of hot (90 – 95°C) glacial acetic acid. Acidification of this technical-purity 1K-salt with a 1% aqueous H_2SO_4 solution yielded a product containing 92 ± 2% of GA (with a yield of 62 – 64% calculated for ~75% glycyrram content in the initial raw material).

In order to obtain a final product of still higher purity, the technical 1K-salt was triply recrystallized from an aqueous ethanol solution. Upon treating this pure 1K-salt with a 1% aqueous H_2SO_4 solution at 100°C, followed by crystallization at 4 – 8°C, we obtained high-purity finely crystalline product containing 97 ± 2% of GA (HPLC data). The yield of crystalline GA amounted to 63 – 67% (calculated for ~75% glycyrram content in the initial raw material).

The content of the parent compound (GA) in the products was determined by HPLC (Zorbax ODS reversed-phase column) using a calibration curve constructed for a standard GA sample of 99.9% purity (obtained by preparative chromatography).

The refined scheme 2 involves simple technological operations and allows a high-purity target glycoside (97 ± 2% GA) to be obtained from either technical glycoside or a commercial glycyrram. The initial scheme 1 provides for obtaining the target glycoside with a purity of 87 ± 2%. The product yields calculated for the initial raw material of 75% purity are approximately equal.

The results of our biological tests showed that crystalline GA possesses moderate immunotropic activity. Injected in a dose of 50 mg/kg, GA reliably stimulated antibody formation in mice: the number of antibody-forming cells (AFCs) increased by a factor of 1.6 ($p < 0.05$). In a greater dose (200 mg/kg), GA showed a tendency to inhibit antibody formation. It was found that, irrespective of the dose, GA inhibits development of the delayed- type cell immunity reaction (see Table 1).

EXPERIMENTAL CHEMICAL PART

The IR spectra were measured on a Specord M-80 spectrophotometer (Germany) using samples prepared as nujol mulls. The electronic (UV) absorption spectra were recorded on Shimadzu UV-365 (Japan) and Specord UV-400 (Germany) instruments using methanol solutions. The optical activity was determined on a Perkin-Elmer Model 241MS polarimeter (Sweden) equipped with a 10-cm tube cell. The melting temperatures were determined in a Boetius heating stage (Germany). TLC analyses were performed on Silufol UV-254 plates and bonded silica gel L (5/40 μm) plates (Czech Republic). The spots were visualized by treating the TLC plates in a 20% phosphotungstic acid solution in ethanol at a temperature of 110 – 115°C.

The ^{13}C NMR spectra were measured on a Bruker AM-300 spectrometer (Germany) operating at a working frequency of 75.5 MHz in the wide-band and nonresonance proton decoupling mode; DMF-d_6 was used as the solvent and TMS, as the internal standard. HPLC analyses were carried out on a Du Pont chromatograph (France) equipped with a 25 cm × 4.6 mm Zorbax ODS reversed-phase column. The column was eluted with a mobile phase of CH_3CN–H_2O–CH_3COOH (35 : 65 : 0.5, vol.%) composition at a flow velocity of 1 ml/min. The UV detector was tuned to λ = 254 nm.

1. Synthesis of Technical Glycoside.

1a. A mixture of 25 g glycyrram, 25 g cation exchanger (KU-2, KU-2-8, KSP-4p, Dowex-50, or Amberlite IR-120) in the H^+ form, and 1 g activated charcoal in 350 – 400 ml of 70 – 75% aqueous ethanol was stirred at room temperature until complete dissolution (~2 h) of the initial salt. Then the exchange resin was separated by filtration and washed with ethanol (30 – 50 ml). The filtrate was evaporated in vacuum at 50 – 60°C. The resulting syruplike glycoside was dried in vacuum at 100°C to constant weight. Yield of technical glycoside (amorphous substance of a yellowish color), 23.5 – 24.5 g (95.9 – 97.9% of theoretical value); $[\alpha]_D^{20}$, +27 ± 2° (*c*, 0.2; 50% EtOH); UV spectrum in 50% C_2H_5OH (ν_{max}, nm): 250 (log ε, 3.93); GA content, 75 ± 2%.

1b. Treatment of a 50-g batch of glycyrram by a procedure analogous to that described in 1a yielded 46.6 – 48.6 g (95.1 – 99.2%) of technical glycoside.

1c. Treatment of a 100-g batch of glycyrram by a procedure analogous to that described in 1a yielded 92 – 94 g (94 – 96%) of technical glycoside.

2. Tripotassium GA Salt (II).

2a. Technical glycoside (23.5 g) was dissolved with stirring and slight heating (≤ 45°C) in 200 ml of acetone. To this solution was added a 10% KOH solution in ethanol to pH 8 – 9. The yellow precipitate of technical 3K-salt was filtered, washed with acetone (50 ml), and dried first in air and then in a thermal box at 100 – 110°C to constant weight. Yield of 3K-salt (yellow powder), 27.4 g (100%); $T_{decomp.}$, 280 – 285°C; $[\alpha]_D^{20}$, +40 ± 2° (*c*, 0.03; H_2O) [13]; $[\alpha]_D^{20}$, +40° (*c*, 0.07; H_2O).

2b. Treatment of a 48.6-g batch of technical glycoside by a procedure analogous to that described in 2a yielded 55.3 g (100%) of technical 3K-salt.

3. Monopotassium GA Salt (III).

3a. Tripotassium salt (27.4 g) was dissolved on heating to 100 – 110°C in 140 – 145 ml of glacial acetic acid. The solution was immediately filtered hot and allowed to crystallize on standing at room temperature overnight. The precipitated 1K-salt was separated by filtration, washed with ethanol, and dried to constant weight at 110 – 120°C to obtain 16.4 g (65.2%) of technical 1K-salt (III); $[\alpha]_D^{20}$, +40 ± 2° (*c*, 0.04; 25% EtOH); IR spectrum (λ_{max}, cm^{-1}): 3600 – 3200 (OH), 1710 (COOH), 1660 [C(11)=O], 1530 – 1500 (COO^-); UV spectrum in 25% C_2H_5OH (λ_{max}, nm): 255 (log ε, 3.85).

3b. A 55.3-g batch of tripotassium salt was dissolved on heating to 90 – 95°C in 550 – 600 ml of glacial AcOH, immediately filtered hot, and allowed to crystallize at room temperature overnight. The white precipitate of 1K-salt was separated by filtration, washed with AcOH and ethanol, and dried to constant weight to obtain 43.0 – 45.6 g (85.2 – 90.3%) of technical 1K-salt (III); $[\alpha]_D^{20}$, +42 ± 2° (*c*, 0.02; 25% EtOH).

The obtained technical 1K-salt (45.6 g) was triply recrystallized from an ethanol – water mixture (3 : 1, v/v) to obtain 32.2 g of pure 1K-salt (63.7% of theoretical value); $[\alpha]_D^{20}$, +50 ± 2° (*c*, 0.03; 25% EtOH). UV spectrum in 50% C_2H_5OH (λ_{max}, nm): 255 (log ε, 4.05); $C_{42}H_{61}KO_{16} \cdot 2H_2O$.

3c. Treatment of a 92-g batch of technical 3K-salt by a procedure analogous to that described in 3b yielded 75 g (100%) of technical 1K-salt. Upon triple recrystallization from an ethanol – water mixture (4 : 1, v/v), the yield of pure 1K-salt is 37.5 g.

4. Synthesis of Glycyrrhizic Acid from Monopotassium Salt.

4a. A mixture of technical 1K-salt obtained as described in 3a (10 g), 5 g cation exchanger (KU-2-8 or KSP-4p) in the H^+ form, and 1 g activated charcoal in 100 ml of 70 – 75% aqueous ethanol was stirred at room temperature until complete dissolution of the initial salt. Then the exchanger resin and carbon were separated by filtration. The filtrate was evaporated in vacuum at 50 – 60°C and the residue was dried in vacuum at 50 – 60°C to constant weight. Yield of technical glycoside (amorphous substance of a yellowish color), 8.2 – 8.4 g; $[\alpha]_D^{20}$, +45 ± 2° (*c*, 0.12; 50% EtOH); UV spectrum in 50% C_2H_5OH (λ_{max}, nm): 250 (log ε, 3.98); GA content, 85 ± 2%; water content, 6.8 – 7.4%.

4b. A mixture of technical 1K-salt (10 g), obtained as described in 3b, was heated for 20 min at 100°C with 100 ml of 1% H_2SO_4, cooled down to room temperature, and allowed to stand in a refrigerator (4 – 8°C) for 48 h. The white finely crystalline precipitate of GA was separated by filtration, washed with cold water to a neutral reaction, and dried at

100°C to constant weight. The dry product was stirred with 100 ml of chloroform, filtered, and dried in vacuum again at 100°C to obtain 8.0 g (83.3% of theoretical value or 64.3% as calculated for the 75% initial glycyrram); m.p., 213 – 215°C; $[\alpha]_D^{20}$, +48 ± 2° (*c*, 0.12; 50% EtOH); UV spectrum in C_2H_5OH (λ_{max}, nm): 250 (log ε, 4.04); parent compound (GA) content, 92 ± 2%; water content, 6.5 – 7.0%.

4c. A mixture of pure 1K-salt (10 g), obtained as described in 3b, was heated for 20 min at 100°C with 100 ml of 1% H_2SO_4, cooled down to room temperature, and allowed to stand in a refrigerator (4 – 8°C) for 48 h. The white finely crystalline precipitate of GA was separated by filtration, washed with cold water to a neutral reaction, and dried at 100°C to constant weight. The dry product was stirred for 1 h in 100 ml of chloroform, filtered, and dried in vacuum again at 100°C to obtain 7.8 g (81.2% of theoretical value or 62.7% as calculated for the 75% initial commercial glycyrram); m.p., 222 – 223°C; $[\alpha]_D^{20}$, +62 ± 2° (*c*, 0.03; EtOH); R_f, 0.2 (chloroform – ethanol, 3 : 1, silica gel L); 0.5 (butanol – acetic acid – water, 6 : 1 : 3, silica gel L); 0.45 (chloroform – ethanol, 3 : 1, Silufol); 0.27 (chloroform – methanol – water, 45 : 10 : 1, silufol). IR spectrum (λ_{max}, cm^{-1}): 3600 – 3200 (OH), 1716 (COOH), 1652 [C(11)=O]; UV spectrum in C_2H_5OH (λ_{max}, nm): 249 (log ε, 4.05); ^{13}C NMR spectrum in DMF-d_6 (δ, ppm): 199.80 (C-11), 178.33 (C-30), 170.74 (C-13), 170.53 (C-6′), 170.39 (C-6″), 127.80 (C-12), 105.38 (C-1′), 104.16 (C-1″), 89.04 (C-3), 83.19 (C-2′), 76.76 (C-3′), 76.59 (C-3″), 76.40 (C-5″), 75.60 (C-5′), 75.60 (C-2″), 72.23 (C-4″), 72.02 (C-4′), 61.76 (C-9), 55.06 (C-5), 48.68 (C-18), 45.42 (C-14), 43.68 (C-20), 28.55 (C-29), 28.27 (C-28), 28.11 (C-23), 23.27 (C-27), 18.61 (C-26), 16.39 (C-25), 16.14 (C-24); parent compound (GA) content, 97 ± 2%; water content, 3.8 – 4.2% [14]; $[\alpha]_D^{20}$, +60.4° (*c*, 1.5; EtOH) [15]; m.p., 228 – 230°C.

EXPERIMENTAL BIOLOGICAL PART

The effect of GA on the humoral immunity response and delayed-type cell immunity reaction was studied on linebred mice by the Gerney – Nordin method modified as described in [16, 17]. The synthesized GA was injected intraperitoneally in a single daily dose of 25, 50, or 200 mg/kg during three days (the first injection was combined with the immunization treatment). The test antigen was represented by goat erythrocytes in the optimum dose [17]. The humoral immunity response was evaluated on the 5th day by determining the number of AFCs in the spleen of immunized test animals. The cell immunity reaction on a delayed-type hypersensitivity model was evaluated by comparing the model foot edema growth in animals of the control and test groups. The "negative" control group contained animals not subjected to the sensitization treatment (receiving only a challenging dose of goat erythrocytes). Mice in the "positive" control group received both the model sensitization and the goat erythrocyte challenge.

Thus, we have developed a method for the synthesis of high-purity (97 ± 2%) glycyrrhizic acid from commercial glycyrram. In parallel, we propose a more efficient technology for obtaining glycyrrhizic acid of purity 92 ± 2% [18].

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REFERENCES

1. R. Pompei, A. Pani, O. Flore, et al., *Experientia*, **36**, 304 (1980).
2. European Patent No. 255420; *Chem. Abstr.*, 109, 116062 (1988).
3. European Patent No. 739627 (1997).
4. H. Abe, N. Ohya, K. Yamamoto, et al., *Cancer Clin. Oncol.*, **23**(10), 1549 – 1555 (1987).
5. K. Kunano, T. Munakata, U. Semba, et al., *Igaku no Ayumi*, **129**(3), 173 – 175 (1984); *Chem. Abstr.*, **101**, 83711k (1984).
6. K. Ito, M. Miyata, M. Kosudo, et al., *Igaku no Ayumi*, **135**(12/13), 1089 – 1990 (1985); *Chem. Abstr.*, **104**, 161940n (1986).
7. O. A. Plyasunova, I. N. Egoricheva, N. V. Fedyuk, et al., *Vopr. Virusol*., No. 5 – 6, 235 – 238 (1992).
8. A. G. Pokrovskii, E. F. Belanov, G. N. Volkov, et al., *Dokl. Akad. Nauk SSSR*, **344**(5), 709 – 711 (1995).
9. S. Ya. Sokolov and I. P. Zamotaev, *Handbook of Medicinal Plants* [in Russian], Meditsina, Moscow (1988).
10. V. A. Manyak, *Abstracts of Papers. The 4th All-Union Congr. of Pharmacists (November 18 – 20, 1986)* [in Russian], Kazan (1986), pp. 247 – 248.
11. USSR Inventor′s Certificate No. 1189453 (1986).
12. L. A. Baltina, V. A. Davydova, T. G. Tolstikova, et al., *Khim.-Farm. Zh*., **25**(3), 45 – 48 (1991).
13. L. A. Baltina, N. G. Serdyuk, L. V. Krasnova, et al., *Khim.-Farm. Zh*., **28**(9), 51 – 54 (1994).
14. French Patent No. 2473526; *Chem. Abstr.*, **96**, 20303m (1982).
15. G. G. Zapesochnaya, E. N. Zvonkova, V. A. Kurkin, et al., *Khim. Prir. Soedin*., No. 6, 772 – 780 (1994).
16. A. J. Cunningham and A. Czenberg, *Immunology*, No. 2, 599 – 600 (1968).
17. F. V. Collins and N. Morrison, *Infect. Immunol.*, **13**(2), 564 – 568 (1976).
18. L. A. Baltina, R. M. Kondratenko, S. R. Mustafina, et al., *Khim.-Farm. Zh*., **35**(1), 38 – 41 (2001).