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ISOLATION AND IDENTIFICATION OF 2-CHLORO-10-(3-DIMETHYLAMINOPROPYL)-PHENOTHIAZINE FROM MUMIYO

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2-Chloro-10-(3-dimethylaminopropyl)-phenothiazine (aminazine) was isolated from the organic extract of mumiyo by distribution chromatography. Its quantitative content was determined by titration.

Key words: organic extract, mumiyo, 2-chloro-10-(3-dimethylaminopropyl)-phenothiazine.

Mumiyo has been used for millenia in Eastern folk medicine as a medicinal substance. Numerous investigations are evidence of its high biological activity and wide spectrum of pharmacological activity [1-3].

About 50 chemical components of inorganic and organic nature are found in mumiyo. Heterocyclic and steroidal compounds in it may play an important role in its high biological activity. The presence in mumiyo of organic acids, amino acids, phospholipids, carbohydrates, phenolic compounds, etc. has been reported [4-8].

We developed methods to isolate and determine qualitatively and quantitatively 2-chloro-10-(3-dimethylaminopropyl)-phenothiazine (aminazine), a medicinal preparation with a broad spectrum of action. We used a sample of mumiyo collected in the Zarafshan highlands of the Republic of Tadzhikistan.

The dry extract of mumiyo obtained by CH_3OH extraction, in contrast with mumiyo itself, is a light brown hygroscopic substance with a distinct odor.

TLC analysis on Silufol UV-254 plates using CH_3OH:H_2O (98:2) showed that the extract contains seven ninhydrin-sensitive components with R_f values 0.12 (**1**), 0.25 (**2**), 0.44 (**3**), 0.54 (**4**), 0.62 (**5**), 0.74 (**6**), and 0.80 (**7**). The chromatogram was sprayed with $PdCl_2$ solution to detect aminazine. Only one spot with R_f 0.62 situated next to the control spot of 2-chloro-10-(3-dimethylaminopropyl)-phenothiazine (standard) was colored red. We used the Ulmann reaction to confirm this observation [9, 10] using in parallel CH_3OH solutions of mumiyo extract and 2-chloro-10-(3-dimethylaminopropyl)-phenothiazine standard and powdered copper. In both instances the solutions were colored green after storage owing to formation of copper chloride in addition to a dimeric product [11].

Chromatography of the CH_3OH extract of mumiyo over a column packed with Sephadex LH-20 produced three fractions: fraction 1 (50 mL), fr. 2 (60 mL), and fr. 3 (60 mL) (Fig. 1).

Aminazine was detected in fr. 1 using color reactions with $PdCl_2$, $FeCl_3$, HNO_3, and H_2SO_4 solutions. TLC on Silufol UV-254 plates using CH_3OH:CH_3COOH:H_2O (95:3:2) revealed that fr. 1 consists of three components including aminazine with R_f 0.91.

This fraction was fractionated twice in succession under the described conditions (Fig. 2). The striped part of the chromatogram indicates aminazine. It was isolated and transformed to the crystalline hydrochloride by addition to gaseous HCl in CH_3OH.

Comparison of reversed-phase HPLC traces of the aminazine isolated from mumiyo and aminazine standard (Fig. 3) indicated that they are identical. UV spectra recorded for aminazine isolated from mumiyo and aminazine standard were identical (CH_3OH, λ_{max}, nm: 262, 310).

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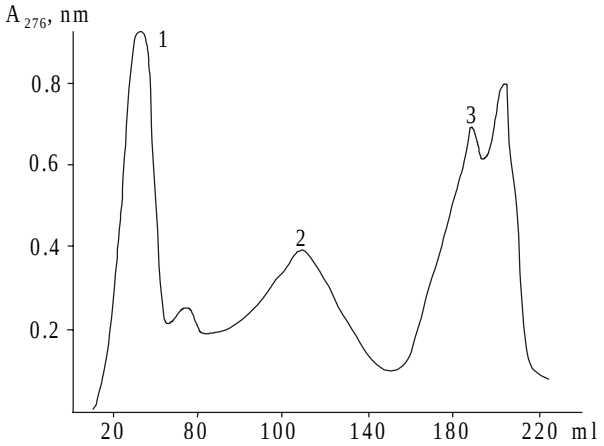

Fig. 1. Elution curves of mumiyo CH_3OH extract over a Sephadex LH-20 column (25-100 µm) using CH_3OH:H_2O (98:2). Elution rate 30 drops/min. Fractions numbers 1, 2, 3.

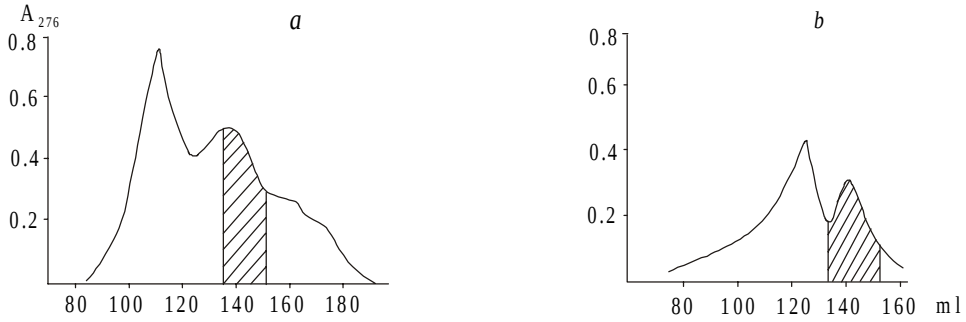

Fig. 2. Successive (*a*, *b*) separation of fraction 1. Elution rate 30 drops/min. Striped fractions were collected.

We also developed a titrimetric method for quantitative determination of aminazine in mumiyo. An analytical amount of the CH_3OH or C_2H_5OH fraction of mumiyo in CH_3COOH with added Hg(II) acetate was titrated by $HClO_4$. The aminazine content was calculated using the formula proposed by us:

$$m = N\ V\ (E_X\ a)/1000\ (A),$$

where m is the mass of aminazine in the analyzed extract in mg; N is the normality of the $HClO_4$ titrant; V is the volume of $HClO_4$ solution used to titrate the analyte in mL; E_x is the equivalent mass of aminazine; and *a* is a factor equal to the ratio of the equivalent mass of chlorine to that of aminazine where a = 0.1. The accuracy of the determination is ±0.5%.

The equivalence point was established visually and potentiometrically.

Potentiometric titration used a Pt electrode in combination with a AgCl electrode. The pH meter (potentiometer) was checked by measuring E for a freshly prepared solution containing:

$$[K_4Fe(CN)_6\ 3H_2O]/[K_3Fe(CN)_6] = 3.8/13.5$$

The emf of the electrodes at 25°C was 275±20 mV. The amount of aminazine was determined using formula (A). Table 1 lists the results from the potentiometric titration. The potentiometric titration curve (Fig. 4) is S-shaped with a sharp jump in the titration corresponding to aminazine neutralization.

TABLE 1. Potentiometric Titration of 2-Chloro-10-(3-dimethylaminopropyl)-phenothiazine (Aminazine) Solution with $HClO_4$ (0.1 N)

Titrant volume, mL (V)	Value of redox potential E (mV)	Change of potential E ΔE/ΔV (mV)
0.05	+475	-
0.1	+452	23
0.2	+495	43
0.3	+674	179
0.4	+697	23
0.5	+705	8
0.6	+709	10
0.7	+708	1
0.8	+704	4
0.9	+703	1
1.0	+703	0

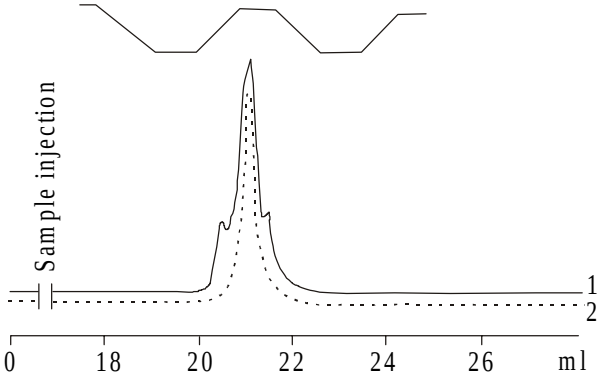

Fig. 3

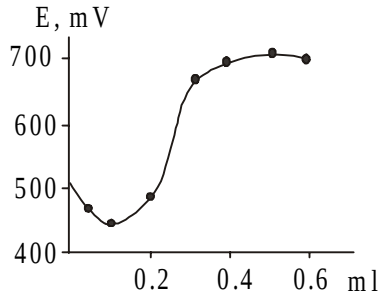

Fig. 4

Fig. 3. Analytical HPLC of aminazine isolated from mumiyo (1) and aminazine standard (2) over a column (5 μm, 10×250 mm). Gradient of CH_3OH and CF_3COOH (0.2%). Elution rate 0.5 mL/min. Recorder rate 0.30 cm/min. Mass of separated sample = 0.005 mg.

Fig. 4. Potentiometric titration by $HClO_4$ (0.1 N) of aminazine from the CH_3OH extract of mumiyo in acetone.

EXPERIMENTAL

TLC was performed on Silufol UV-254 plates; paper chromatography, on FN-14 paper (Germany) using CH_3OH:H_2O (98:2) and CH_3OH:CH_3COOH:H_2O (MAW) (95:3:2) with development by iodine vapor and ninhydrin in acetone (0.5%). Aminazine and other organic components were fractionated chromatographically on an LKB automated chromatograph over a column (2.6×40 cm) packed with Sephadex LH-20 (25-100 μm) at flow rate 30 drops/min using a Uvikord-S at 276 nm. The eluent was CH_3OH:H_2O (98:2). Melting points were determined on a Boetium instrument (Germany). HPLC was carried out on an Altex-340 chromatograph (Beckman, USA) using an Ultrasphere reversed-phase column (5 μm, 10×250 mm) and a Spectroflow-757 detector (Kratos, USA). UV spectra were recorded on a Hitachi-330 spectrophotometer (Japan). Potentiometric measurements were made using a pH 150 digital pH-meter/millivoltmeter and an EPL-02 Pt electrode in combination with an EVL-IM4 AgCl electrode. Solvents were removed in a rotary evaporator at 40-50°C.

Preparation of Mumiyo CH_3OH Extract. Mumiyo (0.6 g) was obtained as before [1], treated with CH_3OH (5 mL), stirred for 2 h, and left at room temperature. The insoluble part was filtered off. The CH_3OH part was evaporated in vacuum in a rotary evaporator. The solid was dried in vacuum to give 0.24 g of light brown hygroscopic powder. Ethanol can also be used as the solvent.

Fractionation. a) The CH_3OH extract of mumiyo (0.040 g) was chromatographed over a column of Sephadex LH-20 by collecting 5-mL fractions. The resulting separated peaks of the fraction (Fig. 1) were combined and evaporated. The solids were lyophilized to give three fractions of amorphous compounds: 0.022 g (**1**), 0.008 g (**2**), and 0.008 g (**3**). Qualitative reactions with $PdCl_2$ solution (cherry-red coloration of the solution), dilute (1:1) HNO_3 (rose color gradually turning to red), and $FeCl_3$ solution (red turning to cherry red after adding water) confirmed the presence of aminazine in fr. 1. Control reactions were conducted in parallel with aminazine standard. Fraction 1, which contained aminazine, was chromatographed on paper using MAW (95:3:2) and developed by ninhydrin in acetone (0.5%). Three spots with R_f values 0.53, 0.67, and 0.73 were detected. The compound with R_f 0.91 is aminazine.

b) Repeated chromatography of the extract of fr. 1 (0.020 g) obtained by method a) isolated two separated fractions of amorphous products: fr. 1 (0.01 g) and fr. 2 (0.008 g).

c) Fraction 2 (b, 0.008 g) was again fractionated (experimental conditions the same as before) to produce aminazine oil (0.003 g), a CH_3OH solution of which was saturated with gaseous HCl and treated with H_2SO_4 to produce a light gray powder with mp 192-195°C (lit. mp 195-198°C) with R_f 0.91 (MAW, 5:3:2). UV spectrum (CH_3OH, λ_{max}): 262, 310 nm.

Titrimetric Determination of Aminazine. The CH_3OH extract of mumiyo (0.016 g) was dissolved in acetone (3 mL), treated with Hg(II) acetate (0.25 mL, saturated solution in glacial CH_3COOH) and methylorange (0.05 mL, saturated solution in acetone), and titrated visually from a microburette by $HClO_4$ (0.1 N) from yellow to orange. The average volume of $HClO_4$ after titrating aminazine three times was 0.45 mL (blank titration used 0.04 mL of $HClO_4$). According to formula (A), the aminazine content in the mixture was 0.00145 g.

Potentiometric Determination of Aminazine. Dry mumiyo extract (0.18 g) was dissolved in acetone (20 mL) containing Hg(II) acetate solution (0.2 mL). The beaker with the solution was placed on a magnetic stirrer. The electrodes were lowered into it. The solution was stirred. The change of redox potential was followed during the titration. The solution was titrated by $HClO_4$ (0.1 N) from a microburette. The change of potential on the scale of the pH meter was noted after each addition of titrant. As the equivalence point (EP) was approached, titrant was added dropwise and measurements were taken every 20-30 sec. The titration end point was determined as the largest jump of potential (Fig. 4). The volume of $HClO_4$ for the titration was 3 mL. According to formula (A), the aminazine content in the mixture was 1.00165 g.

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