MEDICINAL PLANTS

ANGIONORM COMPLEX HERBAL PREPARATION CONSTITUENTS IDENTIFIED BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY

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Angionorm is an original Russian preparation based on the dried aqueous EtOH (25%) extract of a herbal mixture containing horse chestnuts (*Aesculus hippocastanum* L.), licorice roots (*Glycyrrhiza glabra* L.), hawthorn fruit (*Crataegus*), and dog-rose hips (*Rosa canina*). A reversed-phase HPLC-MS-MS procedure with gradient elution was developed to identify the main constituents in extracts of the mixture and each raw material type. Standards helped to identify 15 compounds of various classes (phenolic and hydroxycinnamic acids and their esters, flavonoids, flavonoid glycosides, triterpene saponins). Peaks of hyperoside, liquiritin, â-glycyrrhizic acid, and escin isomers were identified using MRM transitions, elution order, and literature data. Herbal sources of identified constituents were determined. Marker compounds characteristic of each raw-material type in the mixture extract were proposed.

Keywords: Angionorm, HPLC-MS-MS, polyphenolic compounds, flavonoids, saponins.

Angionorm is an original Russian herbal preparation in coated-tablet form based on the dried extract of medicinal plant raw material (MPM) of the same name that includes horse chestnuts (*Aesculus hippocastanum* L.), licorice roots (*Glycyrrhiza glabra* L.), hawthorn fruit (*Crataegus*), and rose hips (*Rosa*) in mass ratio 30:15:20:35, respectively. Angionorm possesses anti-aggregation, angioprotective, analgesic, anti-inflammatory, venotonic, and diuretic properties [1]. The preparation was demonstrated clinically to be safe for use in urgent care and on a regular basis as an antiaggregant to prevent and treat cardiovascular diseases [2].

Identification of the constituents in whole extract of the MPM mixture is critical for validating the pharmacological activity based on the chemical composition.

The extractant for producing the dried extract from the mixture of the four aforementioned MPMs was EtOH (25%). Extracts of each individual MPM source were obtained using other extractants and studied earlier, including by LC-MS [3 – 6]. However, the exact composition of the biologically active constituents co-extracted from the MPM mixture under industrial conditions by 25% EtOH is currently unknown. Existing methods for standardizing Angionorm preparation utilize only qualitative reactions for phenolic compounds and saponins and spectrophotometric determination of total flavonoids. Tandem LC-MS is an effective method for identifying compounds in a complicated multi-component matrix of natural compounds, many of which have similar structures [7 – 10].

The goals of the research were to develop a procedure for separating the constituents of the dried extract from the MPM mixture upon which Angionorm is based, to identify them using HPLC-MS-MS, and to determine the source of these components by analyzing each individual MPM type. The identified biologically active compounds provided a platform for improving the standardization of the herbal

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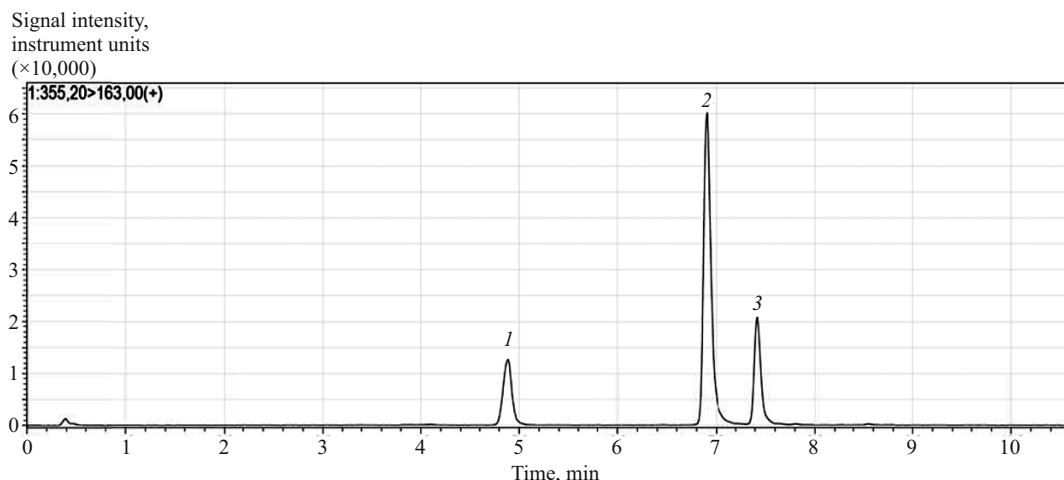* e-mail: peter455@yandex.ru

Signal intensity,
instrument units
(×10,000)

Fig. 1. Chromatogram of chlorogenic acid isomers; detection in MRM mode (*m/z*) 355.20 (163.00: neochlorogenic (*1*), chlorogenic (*2*), and cryptochlorogenic acids (*3*).

preparation and product quality control. In turn, this guaranteed that the biological activity of the preparation was stable.

EXPERIMENTAL PART

Dried extract Angionorm (batch 011015, NPO FarmVILAR, Maloyaroslavets, Russia) and MPM horse chestnuts [11], licorice roots [12], hawthorn fruit [13], and rose hips [14] were used in the work. Dried extract was produced by drying the EtOH (25%) extract of the given MPM mixture.

Compounds were identified using reference standards (RSs) of gallic acid, 2,4-dihydroxybenzoic acid, chlorogenic acid, *p*-coumaric acid, liquiritin, hyperoside, rutin, formononetin, licurazide, quercetin, kaempferol, naringenin, apigenin, glycyram (ammonium glycyrrhizate), β-escin, dihydroquercetin (all Sigma-Aldrich Chemi GmbH, Steinhein, Germany).

MeOH (extra pure, Scharlau, Spain), MeCN (supergradient, Scharlau, Spain), EtOH (96%, Merck, Germany), formic acid (for LC-MS, Merck Millipore, USA), HCl (chemically pure, Reakhim, Russia), conc. NH_4OH (extra-pure, Scharlau, Spain), and deionized H_2O (18.2 MΩ·cm, Millipore Simplicity UV, Merck Millipore, USA) were also used in the work.

Chromatograms were obtained using an LCMS-8040 liquid chromatograph-mass spectrometer in a Nexera system with a triple quadrupole mass spectrometer (Shimadzu, Japan), Prominence SPD-M20A spectrophotometric diode-array detector, gradient elution system, and CTO-20AC column thermostat in the range 4 – 90°C.

Preparation of Angionorm and MPM samples. Dried extract (100 mg) was dissolved in EtOH (10 mL, 25%, prepared from 96% EtOH according to the SP XIth Ed.) in an ultrasonic bath for 30 min and centrifuged at 10,000 rpm.

The supernatant (200 μL) was diluted five times with aqueous formic acid (0.1 vol%).

Milled MPM (4 g) was treated with EtOH (25 mL, 25%), extracted in the dark at room temperature for 48 h, and filtered through blue-ribbon filter paper. The filtrate (1 mL) was diluted five times with aqueous formic acid (0.1 vol%).

Preparation of RS solutions. RS (10 mg) was placed into a 100-mL volumetric flask, dissolved in MeOH (50 mL), and adjusted to the mark with the same solvent. The resulting solution (0.1 mL) was treated with aqueous formic acid (4.25 mL, 0.1 vol%).

Hydrolysis procedure. A solution (5 mL) of Angionorm extract was treated with HCl (5 mL, 1 M) and refluxed on a water bath for 1 h. The resulting solution (200 μL) was treated with aqueous formic acid (700 μL, 0.1 vol%), NH_4OH (10 μL), and H_2O (90 μL).

HPLC-MS-MS mass spectrometric study conditions: Phenomenex Kinetex column, 2.6 × C18, 100 Å (2.6 μm, 150 × 3.00 mm); gradient elution by formic acid (0.1 vol%) in H_2O (A) and in MeCN (B) (0 – 3 min, 2% B; 3 – 37 min, 2 – 44% B; 37 – 41 min, 44 – 95% B; 41 – 46 min, 95% B; 46 – 47.5 min, 95 – 2% B; 47.5 – 50 min, 2% B); column temperature 50°C; mobile phase flow rate 1.1 mL/min; injected sample volume 10 μL; electrospray ionization (ESI); mass (*m/z*) scan range recording positive and negative ions 240 – 2000; ionization source operating parameters: capillary potential 5 kV, heater temperature 400°C, drying gas (N_2) flow rate 20 L/min. Selective MRM transitions were chosen for each RS based on the mass spectrometric studies (Table 1).

RESULTS AND DISCUSSION

Various mobile phases were tested with octadecylsilane sorbent columns Phenomenex Kinetex 2.6 × C18 100 Å (2.6 μm, 150 × 3.00 mm); Agilent Zorbax SB-C18 (5 μm,

150 × 2.1 mm); and Agilent Zorbax SB-C18 Rapid Resolution HD (1.8 μm, 100 × 2.1 mm). The Phenomenex Kinetex 2.6 × C18 100 Å column gave the optimal resolution and efficiency (Table 1), capacity coefficients, and column efficiency for each peak using gradient elution by the formic-acid-modified mobile phase. RSs were analyzed under analogous conditions. Aqueous EtOH (25%) extracts of milled MPM of each of the four specimens were analyzed under the same conditions to determine the plant source. The extracts were produced under conditions simulating the production process. The tandem mass spectrometric detector (triple quadrupole) provided highly selective determination of the extract constituents in the presence of ballast materials by using multiple reaction monitoring (MRM). Preliminary mass spectrometric studies of each RS and literature data were used to select the ionization mode, precursor ions, and im-

pact energies for MRM detection. A total of 15 compounds of various classes in the MPM extracts were identified using RSs and dried extract Angionorm (Table 1).

Several peaks of RS isomers could be detected in dried extract Angionorm by using the MRM transitions given in Table 1. Isomers could be identified in selective MRM detection mode by using literature data for the elution order and relative retention times under chromatography conditions like those reported earlier.

This approach was used to identify the isomers chlorogenic (3-caffeoylquinic), neochlorogenic (5-caffeoylquinic), and cryptochlorogenic (4-caffeoylquinic) acids. The molecular ion $[M + H]^+$ of the isomers gave a characteristic peak with m/z 355.20. The strongest fragment ion for these compounds in positive-ion mode had m/z 163 $[M - \text{quinic acid} + H]^+$ [2]. Detection was made in MRM mode using the

TABLE 1. Angionorm Complex Preparation Constituents Identified Using Reference Standards

No.	Compound	Retention time R_t, min[a]	Capacity coefficient k'	Efficiency NTP (USP)[a, b]	[*]Ionization mode and recorded ions	MRM	Impact energy, eV	Herbal source[c]
				Phenolic acids				
1.	Gallic acid	1.32	1.13	5453	–	m/z 168.85 → m/z 125.15	15	R, H
2.	2,4-Dihydroxybenzoic acid	2.51	3.04	12950	–	m/z 152.75 → m/z 109.25	15	H, C, R, L
3.	Caffeic acid	6.97	10.24	452133	–	m/z 178.70 → m/z 135.15	17	H, R, L
4.	Chlorogenic acid	7.73	11.48	68655	+	m/z 355.20 → m/z 163.00	14	H, R, L
5.	p-Coumaric acid	9.55	14.23	591289	–	m/z 162.70 → m/z 119.30	17	H, C, R, L
					–	m/z 162.90 → m/z 93.40	36	
				Flavonoids – glycosides				
6.	Liquiritin	13.23	20.39	154767	+	m/z 419.25 → m/z 256.95	12	L
7.	Hyperoside	13.86	21.41	210433	+	m/z 465.00 → m/z 302.95	15	R, H
8.	Rutin	14.06	21.73	226534	+	m/z 611.25 → m/z 302.85	23	H, C, L
					+	m/z 611.25 → m/z 465.20	14	
9.	Licurazide	18.34	28.66	411429	+	m/z 551.05 → m/z 256.90	21	L
				Flavonoids – aglycons				
10.	Luteolin	14.49	22.35	256065	+	m/z 286.85 → m/z 152.90	34	C
11.	Quercetin	19.21	30.03	140544	–	m/z 300.80 → m/z 151.20	22	R, H, C
12.	Kaempferol	19.63	30.75	438749	+	m/z 287.10 → m/z 152.90	33	H, C
13.	Formononetin	24.96	39.41	442862	+	m/z 269.15 → m/z 197.10	41	L
				Triterpene saponins				
14.	Glycyrrhizic acid	30.18	47.81	836451	+	m/z 823.50 → m/z 453.20	29	L
15.	β-Escin	31.47	49.91	1194165	+	m/z 1131.55 → m/z 505.40	28	C
					+	m/z 1131.55 → m/z 435.05	39	

[a] Average of three independent determinations.
[b] Calculated according to 621 Chromatography USP 37 (2014).
[c] H, hawthorn fruit; C, horse chestnuts; L, licorice root; R, rose hips.
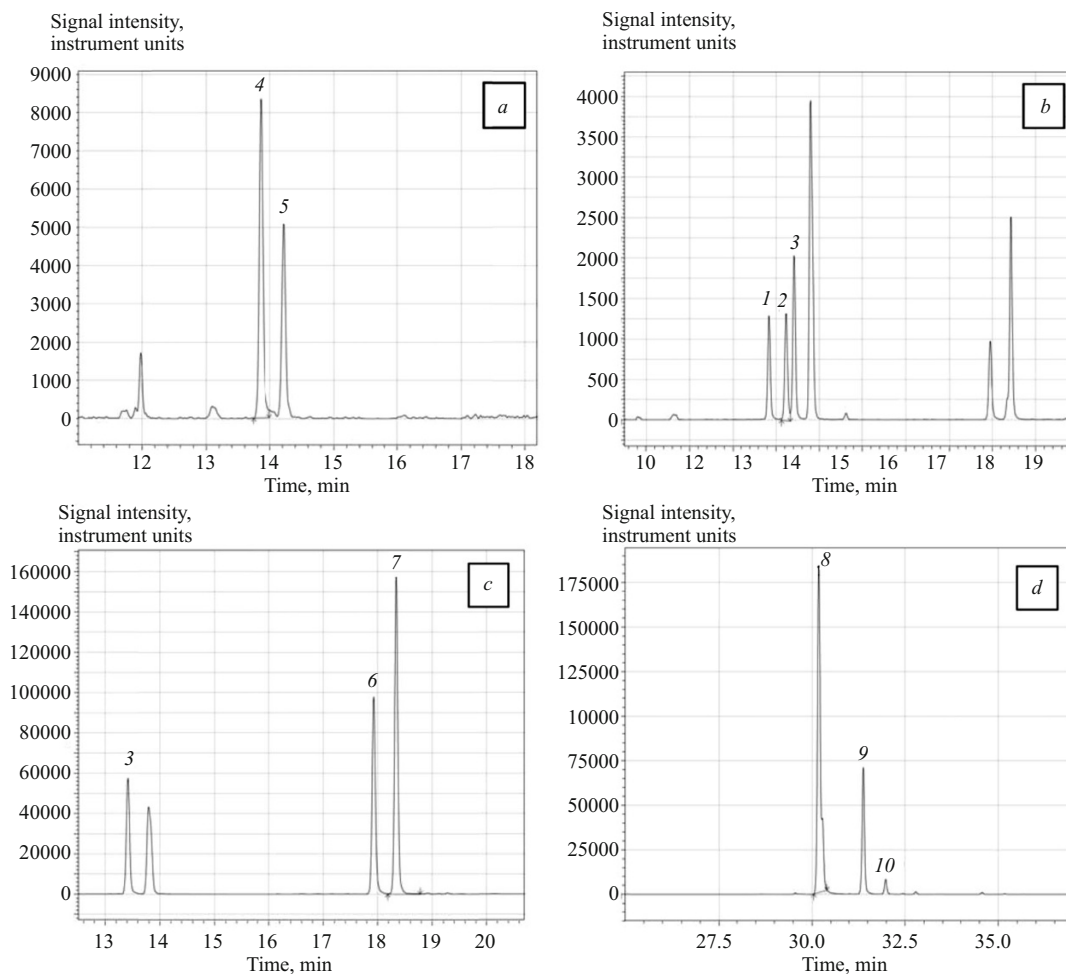[*] Negative (–) and positive (+) ions.

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Fig. 2. Chromatograms of isomeric compounds determined using MRM transitions given in Table 1: hyperoside (*4*) and its isomer isoquercetin (*5*) (*a*); liquiritin (*2*) and its isomer neoliquiritin (*1*) and licurazide isomer liquiritin apioside (*3*) (*b*); licurazide and its isomers (*7*), liquiritin apioside (*3*), and isoliquiritin apioside (*6*) (*c*); 18β-glycyrrhizic acid and its isomers (*8*), 18α-glycyrrhizic acid (*9*), and licorice-saponins H2 or K2 (*10*) (*d*).
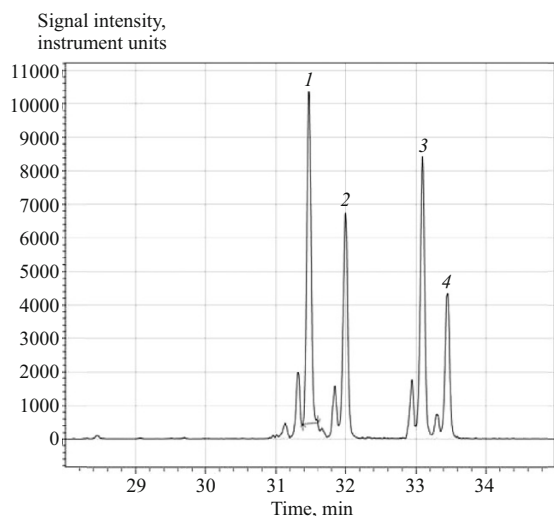

Fig. 3. Chromatogram of main chestnut triterpene saponins; detection in MRM mode (*m/z*) 1131.55 → 505.40 and 1131.55 → 435.05; escin Ia (*1*), escin Ib (*2*), isoescin Ia (*3*), and isoescin Ib (*4*).

transition (*m/z*) 355.20 → 163.00. An Agilent Zorbax SB-C18 Rapid Resolution HD column (1.8 μm, 100 × 2.1 mm) in gradient mode at flow rate 1 mL/min was used to create chromatographic conditions like those reported before [8] and to obtain the optimum separation of chlorogenic acid isomers. Neochlorogenic acid eluted before chlorogenic acid in the chromatogram; cryptochlorogenic acid, after it, as was found before [8, 9] (Fig. 1). An analysis of the MPM sources showed that these isomers were present in hawthorn fruit and rose hip extracts. Chlorogenic acid was also found in licorice root extract. It is noteworthy that the neochlorogenic acid peak was stronger for hawthorn fruit extract; chlorogenic acid, in Angionorm extract (Fig. 1).

Figure 2 (a-d) shows chromatograms of Angionorm extract under the optimal conditions for various selective MRM detection modes. Peak 4 in the chromatogram of Fig. 2*a* belonged to hyperoside (quercetin-3-*O*-β-D-galactoside). Peak 5 could be identified as isoquercetin (quercetin-3-*O*-β-D-glu-
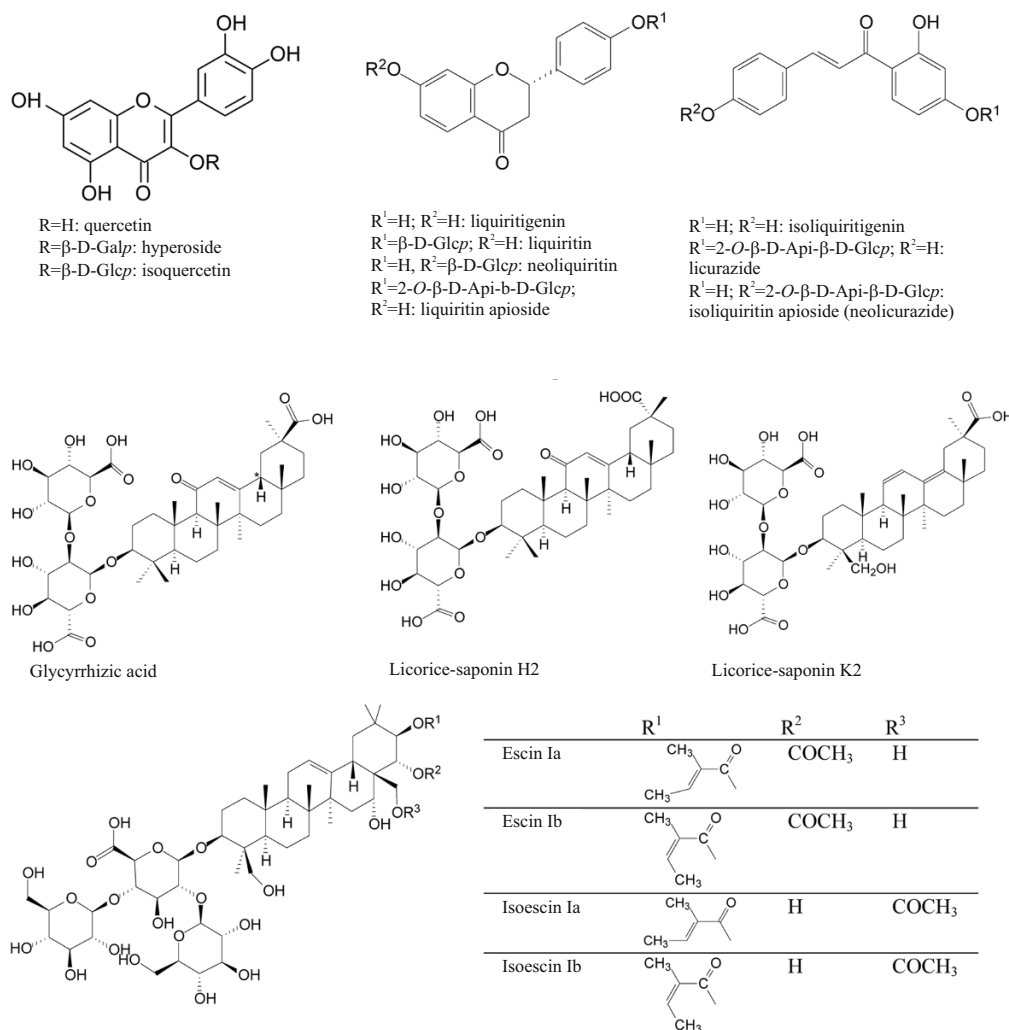
Fig. 4. Structural formulas of identified isomeric compounds.

coside). The ion with *m/z* 302.95 [M – Gal + H]$^+$ and [M – Glc + H]$^+$, where Gal and Glc are galactose and glucose monosaccharide residues, respectively, was a characteristic fragment ion for both compounds. The MRM transition (*m/z*) [M + H]$^+$ 465.00 → 302.95 was used for detection. The relative retention time of isoquercetin vs. hyperoside was 1.027, which agreed with the literature [4, 5, 15]. Peaks for hyperoside and isoquercetin were also observed in chromatograms of rose hip and hawthorn fruit extracts.

The MRM transition (*m/z*) 419.25 → 256.95 detected six peaks (Fig. 2*b*), including a peak for liquiritin (liquiritigenin-4′-*O*-β-D-glucoside, peak 2), which was identified using the RS. Four of these six peaks had retention times that coincided with peaks of compounds that were characterized by another MRM transition (*m/z*) 551.05 → 256.90 (Fig. 2*c*). Peak 7 was identified using a RS as licurazide (isoliquiritigenin-4′-*O*-apiosylglucoside). The molecular ion [M + H]$^+$ of liquiritin and neoliquiritin monoglycosides (liquiritigenin-7-*O*-β-D-glucoside) had a characteristic *m/z* value of 419.25 whereas a molecular ion [M + H]$^+$ with *m/z* 551.05

was characteristic for biosides such as liquiritin and isoliquiritin apiosides (isoliquiritigenin-4-*O*-β-D-glucoside) [3, 16 – 18]. The ion with *m/z* 256.9 could be formed via fragmentation of flavonoid and chalcone monoglycosides (liquiritigenin, isoliquiritigenin) [M – Glc + H]$^+$ [3, 16, 17, 19] and biosides [M – Glc – Api + H]$^+$, where Api is the apiose residue. The peak for the liquiritin isomer (peak 1) could be identified as neoliquiritin according to the characteristic MRM transition and retention time [17, 20] (Fig. 2*b*). Licurazide isomers could be determined from two peaks as liquiritin apioside (liquiritigenin-4′-*O*-apiosylglucoside, peak 3) and isoliquiritin apioside (isoliquiritigenin-4-*O*-apiosylglucoside, neolucurazide, peak 6) according to retention times under analogous chromatographic conditions [19] and UV spectra obtained with a diode-array detector (isoliquiritin, chalcone, characteristic λ$_{max}$ 372 nm; liquiritin, flavanone, λ$_{max}$ 276 nm). The peak for the licurazide isomer with R_1 = 13.80 min (Fig. 2*b* and 2*c*) could not be identified. A comparison of the results with the literature [21] suggested that this peak belonged to a liquiritigenin diglycoside (liqui-

ritigenin-4′-O-glucoside-7-O-apioside) or bioside (liquiriti-genin-7-O-apiosylglucoside).

18β-Glycyrrhizic acid (Fig. 2*d*, peak 8) and two of its isomers could be detected by using the MRM transition (*m/z*) [M + H]$^+$ 823.50 (453.20 [M – (2·GlcU – H$_2$O) + H]$^+$, where GlcU is a glucuronic acid residue. A peak with R_t = 31.45 min (peak 9) was most probably 18α-glycyrrhizic acid. This peak and the peak for 18β-glycyrrhizic acid were observed in the solution of glycyram RS. The retention time relative to β-glycyrrhizic acid agreed with the literature [22] and confirmed this hypothesis. The peak with R_t = 32.00 min (peak 10) was assumed by analogy with the literature [3] to be either licorice-saponin H2 or K2. The aforementioned peaks were also observed in chromatograms of licorice root extract. Figure 3 presents the formulas of the isomers.

Escin was a characteristic constituent of horse chestnuts [6]. Escin is a mixture of triterpene saponins, the main ones of which are the four isomers escin Ia, escin Ib (the mixture is β-escin), isoescin Ia, and isoescin Ib (the mixture is α-escin) (Fig. 3) [6]. These compounds were identified using detection by MRM transitions (*m/z*) 1131.55 → 505.40 [M – aglycon – H$_2$O]$^+$ and 1131.55 → 435.05 (strongest fragment ion in the obtained mass spectrum) and elution order under analogous chromatographic conditions (Fig. 4) [6].

Thus, the developed HPLC-MS-MS procedure was based on effective separation of constituents in a complicated MPM mixture and detection using secondary ionization and fragmentation of molecular ions to identify several isomers of biologically active compounds with similar structures.

Marker compounds characteristic of each type of MPM in the mixture extract could be selected based on the results. Thus, liquiritin, licurazide, formononetin, and β-glycyrrhizic acid (the dominant amount) were specific for the EtOH (25%) extract of licorice root. β-Escin was a marker of horse chestnut extract. The peak intensities in extracts of hawthorn fruit and rose hips should be considered when determining the indicator constituents. Gallic acid, the peak area of which in rose hip extract was 65.8 times greater than in hawthorn fruit extract, was chosen as the marker for rose hips. The peak areas of chlorogenic and neochlorogenic acids in hawthorn fruit extract were 8.2 and 25.9 times, respectively, greater than in rose hip extract. Isoquercetin was detected only in hawthorn fruit extract. This enabled these three compounds to be selected as markers for hawthorn fruit.

A comparison of chromatograms of MPM mixture extract before and after acid hydrolysis showed a substantial weakening of peaks in the range of diglycosides and glycosides and strengthening of those for the aglycons. The peak area of quercetin increased by 3.41 times. A peak for naringenin appeared and was identified using an RS and MRM (*m/z*) 271.05 → 119.30. This flavonoid was not detected in the free state in the mixture extract, suggesting that only naringenin glycosides were extracted. The carbohydrate moieties of the glycosides were not characterized.

Thus, the main constituents in the extract of a mixture of four MPMs, i.e., horse chestnuts, licorice roots, hawthorn fruit, and rose hips, or the basis of Angionorm preparation, were identified. Biologically active compounds characteristic of each MPM type included in the preparation were found. Indicator constituents specific for each MPM type that could be used to analyze and standardize the extract and Angionorm herbal preparation were determined. A modern standardization system for drug substances and dosage forms that guaranteed stable pharmacological effects was developed based on the results.

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