IDENTIFICATION AND QUANTITATIVE DETERMINATION OF FENOZAN ACID IN BIOLOGICAL OBJECTS BY CHROMATOMASS SPECTROMETRY

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Fenozan acid (4-hydroxy-3,5-di-*tert*-butylphenylpropionic acid, I) belongs to the class of sterically hindered phenols. This synthetic antioxidant exhibits all the general properties of natural antioxidants [1 – 3].

In the stage of preclinical investigation of the parmacokinetics of fenozan acid, we developed methods for the identification of I in a complex multicomponent biological material and for the quantitative determination of this compound in the presence of endogenous compounds by means of chromatomass spectrometry.

EXPERIMENTAL PART

Analytical equipment. The analyses were performed using a Hewlett-Packard gas chromatography – mass spectrometry (GC – MS) system comprising an HP Model 5980 Series II gas chromatograph, an HP Model 5971A mass selective detector, and an HP Model 59970C data processing station.

The samples were separated on a 12 m × 0.2 mm capillary column filled with an HP-1 methylsilicon phase (0.33 μm). The carrier gas (helium) was supplied at an input pressure of 3 psi (21 kPa). The gas flow rate was 40 ml/min at the injector slot and 1 ml/min in the spacer system. The samples were applied in the splitless/split mode, with the split valve switched on 1 min upon sample introduction into the injector. The injector was operated at a temperature of 250°C.

Fenozan acid was quantitatively determined after extraction from biological fluids by diethyl ether. Compound I was determined in the form of fenozan acid methyl ether (II) upon the stage of preliminary derivatization with diazomethane.

The quantitative determination of II was performed in the following programmed temperature regime: initial column temperature $T = 75°C$ was maintained for 2 min after sample injection, then increased to 280°C at a rate of 50 K/min, and maintained constant until the end of the program. The mass-selective detector (MSD) was connected to the capillary column by a direct input interface operated at 280°C. The MSD operates by the principle of electron impact ionization at an electron energy of 70 eV. Ions were separated by the mass to charge ratio (m/z) in a 203-mm-long quadrupole mass analyzer. The ions were detected in the mass range from 10 to 650 amu.

Standard solutions. The standard plasma solution was prepared from a lyophilized dry plasma by adding distilled water and thoroughly stirring, standing, and filtering it through a Capron fabric.

The standard stock solution of I with a concentration of 1 mg/ml was prepared by dissolving an accurately weighed amount of air-dried fenozan acid in methanol. The standard marker solutions were obtained by diluting the stock solution to marker concentrations ranging from 10 to 1000 μg/ml (Table 1). The calibration solutions were prepared by adding 100 μl of each marker solution to 1 ml of the plasma so as to obtain solutions of I with concentrations from 1 to 100 μg/ml (C_{pl}). Three calibration solutions were prepared for each concentration. A "zero" solution contained 100 μl of pure methanol in the plasma. After the marker introduction, all solutions of I in the plasma were allowed to equilibrate by standing for not less than 1 h at 20°C.

Fenozan acid isolation. Compound I was isolated from calibration solutions and biological fluids by extracting with ether from solutions acidified to pH 1 – 2. The samples of biological fluids with the native compound and/or its metabolites were stored at –18°C and defreezed immediately before analysis; the samples were equilibrated for not less that 1 h at room temperature prior to extraction.

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TABLE 1. Calibration Solutions of Fenozan Acid in the Plasma

Sample	C_{in}, mg/ml	V_{in}, ml	V_{tot}, ml (MeOH)	C_{mark}, μg/ml	A C_{pl}, μg/ml	B C_{GC}, ng/μl		C m (I), ng	
						200 μl	25 μl	200 μl	25 μl
1	1	–	–	1000	100	500		300	
2	1	3.5	5	700	70	350		210	
3	1	2.5	5	500	50	250		150	
4	1	1.0	5	200	20	100		60	
5	1	0.5	5	100	10	50		30	
6	1	0.25	5	50	5	25		15	
7	1	0.10	5	20	2	10		6	
8	0.1 μg/ml	0.5	5	10	1	5		3	
9	0.1 μg/ml	0.25	5	5	0.5	2.5	20	1.5	12
10	0.01 μg/ml	0.5	5	1.0	0.1	0.5	4	0.3	2.4
11	0.01 μg/ml	0.25	5	0.5	0.05	0.25	2	0.15	1.2
12	0.001 μg/ml	0.5	5	0.1	0.01	0.05	0.4	0.03	0.24

Notes. A, for a marker volume of 10 μl and plasma volume 1 ml; B, for a reconstructing solution volume of 200 μl or 25 μl; C, for a sample volume of 0.6 μl introduced into the chromatograph.

The samples of biological fluid and calibration solutions (1 ml) were placed in extraction tubes, acidified by adding an equal volume of 0.1 M HCl, and triply extracted with 2.5 ml of diethyl ether. Upon every extraction, the mixture was centrifuged (1 min at 3000 rpm) to separate the phases. Then the ether extracts were combined and the solvent was evaporated in a flow of air at 40°C.

Methylation and reconstruction. To each dry extract was added 1 ml of an ether solution of diazomethane and the mixture was allowed to stand overnight in flasks with ground-glass stoppers. Upon derivatization, the solvent was evaporated (air flow, 40°C) and the dry residue was reconstructed by adding 50 – 200 μl of a dodecane (15 ng) solution in methanol (internal chromatographic standard); an

TABLE 2. Efficiency of Fenozan Acid Extraction from Plasma

C_{pl}, μg/ml	Extraction stage	Percentage extraction	Extraction yield, %
100	1	70	
	2	21	
	3	4.8	100.1
	4	2.3	
	5	1.2	
	6	0.8	
10	1	75.2	
	2	21.8	99.7
	3	2.7	

TABLE 3. Analysis of Fenozan Acid Calibration Solutions in the Plasma

Sample	C_{pl}, μg/ml	C_{GC}, μg/ml	Q_1	Q_2	Q_3
1	100	500	338	340	351
2	70	350	238	242	242.7
3	50	250	170.7	168.3	190
4	20	100	58.5	51.8	56.4
5	10	50	31.12	39.3	31.6
6	5	25	12.4	10.02	13.6
7	2	10	4.44	4.52	2.66

0.6 – 1.0 μl volume of this solution was immediately introduced into chromatograph (C_{GC}).

GC – MS measurements. Quantitative determination of I was performed in the selective ion monitoring (SIM) mode by detecting ions with $m/z = 277$ (quantitative analysis) and 292 and 219 (identification support); the internal standard (dodecane) was analyzed at $m/z = 170$ (quantitative analysis) and 85 (identification support).

The calibration plot was constructed as the ratio of areas under the analytical peak ($m/z = 277$) and the internal standard peak ($m/z = 170$) $Q = S_{277}/S_{170}$ versus the concentration of I in the plasma. The calibration calculations were performed on a personal computer using a program providing for determination of the calibration equation coefficients, dispersion, and the linear regression coefficient (Casio, Japan).

The procedure for the quantitative analysis of I in biological fluids includes the following sequence of stages: liquid-liquid extraction of I by ether from acid solution; conversion of the acid into the methyl ester by reaction with diazomethane; separation on a column; registration with MSD. The characteristic retention times are as follows: I, 6.44 min; II, 6.02 min; internal standard, 3.55 min.

The extraction efficiency was assessed by comparison with a control sample prepared by adding an accurately weighed amount of I (100 and 10 μg) to an ether extract of the standard plasma. The results are summarized in Table 2.

The fragmentation of I and II molecules under ion-impact ionization conditions was as follows (main peaks and their relative intensities with respect to the base peak, %):

TABLE 4. Accuracy of the Proposed Method of Fenozan Acid Determination in the Plasma

n	Q	C_i	$\overline{C}_{det}$	$\overline{C}_{det} - C_i$	S	$C_{in} - C_{det}$	ΔC
1	13.157	4.74		0.83		0.74	18.5
2	8.93	3.70		0.21		0.30	7.5
3	9.57	3.86		0.05		0.14	3.5
4	9.80	3.92	3.91	0.01	0.455	0.08	2.0
5	7.44	3.34		0.57		0.66	16.5
6	8.56	3.61		0.30		0.39	9.75
7	11.01	4.21		0.30		0.21	5.25

I: 263(100), 57(46), 278(20), 219(16), 264(16), 147(15), 128(14), 203(5);

II: 277(100), 57(50), 147(42), 292(41), 219(28), 278(20), 203(15), 161(15).

Reproducibility of the GC – MS measurements was evaluated using a standard solution containing 12.5 ng/μl of compound II (C_{GC} of the chromatographed sample) and 10 ng/μl of the internal standard. For seven repeated determinations, the metrological characteristics were as follows: $\overline{Q} = 16.54$; $S = 1.52$; $S_r = 0.09$; $\varepsilon = 1.4$; confidence interval 16.5 ± 1.4; $W = 9.2$ %.

Reproducibility of the whole analytical procedure was evaluated using a standard solution containing 4 mg/ml of fenozan acid in the plasma (C_{pl}) For seven repeated determinations, the metrological characteristics were as follows: $Q = 9.8$; $S = 1.85$; $S_r = 0.19$; $\varepsilon = 1.7$; confidence interval 9.8 ± 1.7; $W = 17$ %.

Here, W is the variation coefficient, S is the mean square deviation, $S_r = S/\bar{x}$; and $\varepsilon = t_\alpha S\sqrt{n}$, where t_α is the Student coefficient for $n = 7$ and $\alpha = 0.95$.

Table 3 shows the results of the calibration. The general regression equation is as follows: in the concentration range from 100 to 0.5 μg/ml, $C_{pl} = 0.245Q + 1.515$ μg/ml (linear regression coefficient $r = 0.998$); in the concentration range from 0.5 to 0.01 μg/ml, $C_{pl} = 0.097Q - 0.0051$ μg/ml (linear regression coefficient $r = 0.989$).

For verification of the overall procedure, an independently prepared sample representing a standard solution of I in the plasma with a concentration of 4 μg/ml was analyzed together with a series of calibration solutions. Table 4 presents data on the concentration of I determined by the calibration plot and calculated by the regression equation $\overline{C}_{det} = \sum \frac{C_i}{7}$.

Conventional statistical processing of the results showed evidence of their reliability and high reproducibility (for $\alpha = 0.95$): the maximum single deviation of the average value of C_{det} from the true concentration was 18%; the deviation calculated for the average ΔC value is 2%. Thus, the proposed method for the identification and quantitative GC – MS determination of fenozan acid in biological objects ensures high sensitivity and good reproducibility.

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