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MEDICINAL PLANTS

QUANTITATIVE DETERMINATION OF RUTIN IN BIOLOGICAL SAMPLES OF RATS UPON ADMINISTRATION OF *Primula veris* L. HERBAL DENSE EXTRACT

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Rutin contents in biological samples of blood plasma, cardiomyocytes, and mitochondria of cardiomyocytes in rats after administration of *Primula veris* L. dense herbal extract were determined using a quantitative HPLC method. The optimum conditions for quantitative determination of rutin were KH_2PO_4 buffer (50 mM, pH 4.6), 82:18% (v/v) aqueous to organic phase ratio, 30°C, detection at 205 nm, flow rate 1 mL/min, 12 MPa pressure in chromatographic system, and retention time 6 – 6.5 min. Rutin was found to enter the blood and distribute into the heart, in which its concentration was 5.34 times higher than in blood, and to penetrate mitochondria, in which its concentration was 1.7 times higher than in plasma and 3.18 times lower than in the heart.

Keywords: rutin, HPLC, mitochondria.

Chronic heart failure (CHF) currently comprises a large fraction of heart pathologies with the number of afflicted patients consistently rising [1, 2]. The effectiveness of treatments for this pathology is still unsatisfactory despite definite progress in pharmacotherapy. Therefore, new approaches to CHF therapy must be found. A promising contemporary approach involves protection of cardiomyocytes from free-radical damage [3, 4]. Oxidative stress is known to play a key role in the pathogenesis of CHF [5]. The reactive oxygen species initiating it are formed in most instances by leakage of electrons from the mitochondrial electron-transport chain at the level of complexes I and III [6].

Most phytopreparations contain biologically active compounds with antioxidant activity [7]. Thus, the search for compounds to treat CHF among medicinal plants can be considered reasonable. A study of the cardioprotective properties of dense herbal extract of *Primula veris* L. or *P. officinalis* (L.) Hill (DHEPV) from the flora of the European part of the RF using experimental CHF in rats showed a positive effect on myocardium contractility [8]. DHEPV was found to contain phenolic compounds, among which flavonoids, including polymethoxylated ones, were found [9]. They acted as traps for superoxide radical and hydrogen peroxide, thereby limiting further formation of more toxic products. It seemed important to detect the active constituents of DHEPV in mitochondrial cardiomyocytes as a potential target of its action. An analysis of the main classes of the biologically active compounds showed that flavonoids, namely rutin, which was proposed as the main marker compound for standardizing DHEPV, were dominant. The rutin content in DHEPV was 6 – 7%.

The goal of the present work was to determine quantitatively the rutin content in DHEPV and rat plasma, heart, and mitochondrial cardiomyocytes.

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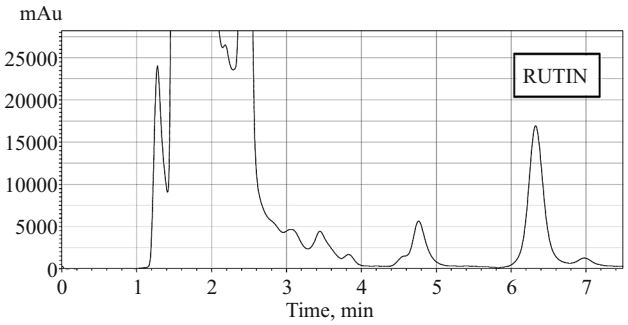

Fig. 1. Chromatogram of rutin standard solution in biological sample. Along the abscissa, time (min); along the ordinate, optical density units (mAu).

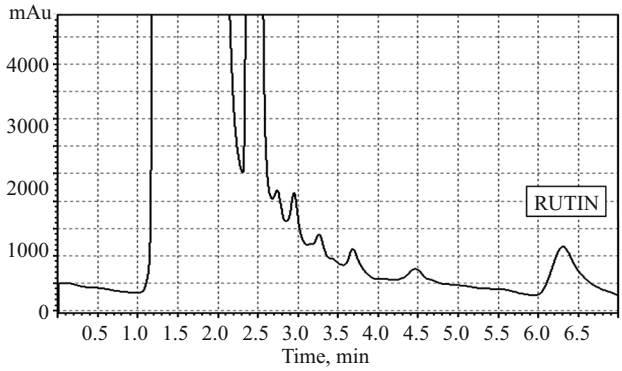

Fig. 3. Chromatogram of rutin in rat heart homogenate upon peroral administration of DHEPV at a dose of 300 mg/kg, corresponding to 20 mg of rutin per kg. Along the abscissa, time (min); along the ordinate, optical density units (mAu).

EXPERIMENTAL PART

The reagents KH_2PO_4 (pure, Russia), K_2HPO_4 (analytically pure, Russia), MeCN (UV-200, Spain), NaCl (chemically pure, Russia), EDTA (analytically pure, Russia), HEPES buffer (Belgium), and rutin standard (Sigma-Aldrich, USA) were used in the work.

The studies used a Shimadzu liquid chromatograph (Japan). Detection was made by a UV detector at 205 nm. The separation used a SUPELCOSIL LC-18 column (5 μm; 150 mm × 4.6 mm).

The experiments used 10 male rats (250 – 300 g). The animals were obtained from Rappolovo Laboratory Animal Nursery (Leningrad Oblast) and were kept under Volgograd SMU vivarium conditions. Care and manipulations of animals complied with recommendations of Russian Federation National Standard GOST R-53434-2009, "Good laboratory practice rules," requirements of the *European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes* (Strasbourg, June 22, 1998), MH RF Order No. 199n of Apr. 1, 2016, "On approval of good laboratory practice rules," and GOST 33044-2014. Animal groups were formed as follows: 1, test (n = 5), which were administered perorally DHEPV at a dose of 300 mg/kg corresponding to 20 mg of rutin per kg in a volume of 0.2 mL per 100 g of mass; 2, control (n = 5), which received distilled H_2O in the same manner as the test group. The rutin content in biological samples was determined 1.5 h after DHEPV administration. Animals were decapitated. Blood was collected. The heart was excised. An anticoagulant of sodium citrate (5%, 1:10) was used. Hearts were placed in normal saline (0.9% NaCl), thoroughly rinsed of blood, cleaned of vessels and fatty tissue, again rinsed with normal saline, weighed, minced with scissors in a Petri dish, and homogenized in a Potter—Elvehjem tissue homogenizer (glass homogenizer with a Teflon pestle) with added sucrose isolation medium (1:5 ratio) containing mannitol (220 mM), sucrose (100 mM), EDTA (1 mM), KH_2PO_4 (4 mM), and HEPES (20 mM) at pH 7.3.

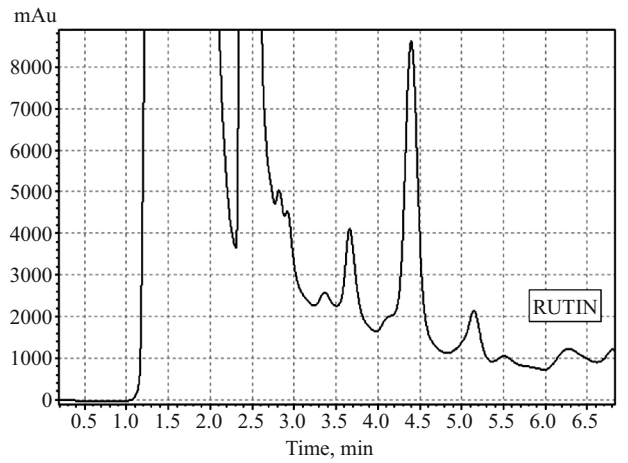

Fig. 2. Chromatogram of rutin in rat blood plasma upon peroral administration of DHEPV at a dose of 300 mg/kg, corresponding to 20 mg of rutin per kg. Along the abscissa, time (min); along the ordinate, optical density units (mAu).

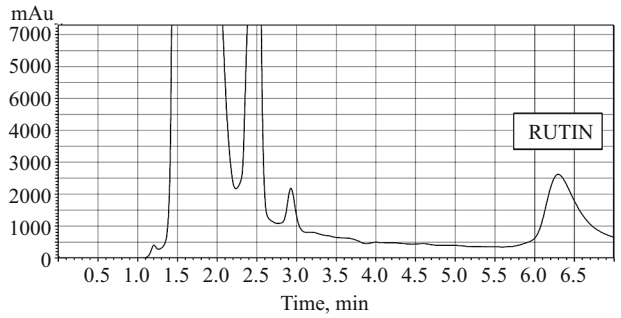

Fig. 4. Chromatogram of rutin in rat mitochondria upon peroral administration of DHEPV at a dose of 300 mg/kg, corresponding to 20 mg of rutin per kg. Along the abscissa, time (min); along the ordinate, optical density units (mAu).

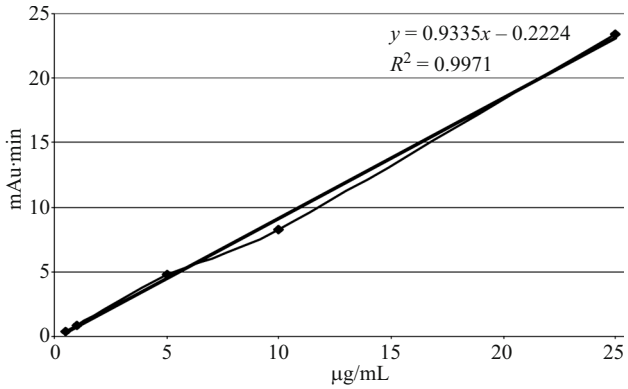

Fig. 5. Dependence of area under the chromatographic peak on rutin concentration in biological sample. Along the abscissa, rutin concentration (μg/mL); along the ordinate, area under chromatographic peak (mAu·min).

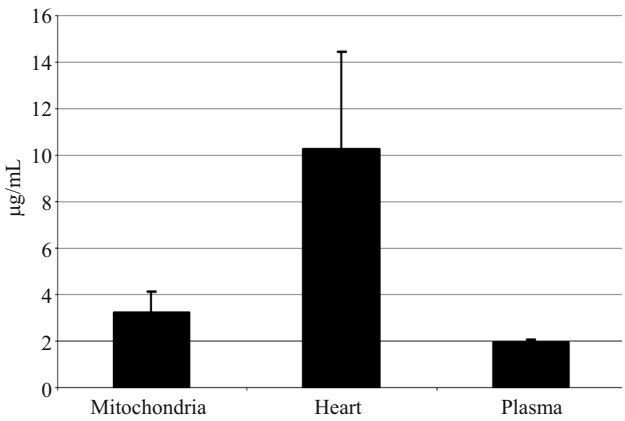

Fig. 6. Rutin content in rat biological samples after peroral administration of DHEPV at a dose of 300 mg/kg (20 mg of rutin per kg). Along the ordinate, rutin concentration (μg/mL).

Mitochondria were obtained by the standard method of differential centrifugation [10].

The test compound was identified and its concentration was calculated using absolute standards. The dependence of peak areas on rutin concentration was determined by regression analysis. Results were statistically processed using Microsoft Excel software.

RESULTS AND DISCUSSION

Figures 1 – 4 show typical chromatograms of rutin in the biological samples.

The chromatography and detection conditions were optimized for several parameters. Selectivity was optimized by studying the chromatographic properties of rutin with the buffer acidity varying from 3 to 6 in steps of 0.5 pH units. The effect on the method selectivity was also studied with varying ratios of eluent aqueous and organic phases. An increase of the MeCN content in the mobile phase from 15 to 25% reduced the retention time of rutin and caused the rutin peak to coalesce with endogenous peaks. The method sensitivity was maintained by lowering the detection wavelength. The analytical parameters of the rutin peak (peak height and area) were studied at wavelengths from 200 to 370 nm. The maximum sensitivity was achieved at 205 nm.

The optimum conditions for quantitative determination of rutin were buffer with KH_2PO_4 (50 mM, pH 4.6), aqueous to organic (MeCN) phase ratio 82:18% (v/v), 30°C, detection wavelength 205 nm, flow rate 1 mL/min, and chromatography system pressure 12 MPa. The retention time was 6 – 6.5 min under these conditions [11].

Rutin was extracted from biological samples by 95% EtOH in a 1:1 ratio. Samples were shaken for 10 min in a horizontal shaker and centrifuged for 15 min at 10,000 rpm in an Eppendorf centrifuge. The supernatant liquid was collected and injected into the injector (20 μL loop).

An analogous extraction using MeCN led to reduced sensitivity and selectivity.

Quantitative determination used a concentration range from 500 ng/mL to 25 μg/mL. The resulting calibration curve was linear with a correlation coefficient (R^2) of 0.997 (Fig. 5).

The method was validated according to the *Guideline on Bioanalytical Method Validation* (EMEA 2012) [12].

The method sensitivity (detection limit) for rutin was 100 ng/mL; limit of quantitation, 500 ng/mL. The average error of a measurement (precision) was <8%. The measurement accuracy was (100 ± 10)%.

The average rutin content in blood plasma samples from experimental animals was 1.92 μg/mL; in heart cells, 10.24 μg/mL; in mitochondria, 3.21 μg/mL (Fig. 6).

Thus, rutin entered the blood of the animals and distributed in the heart after peroral administration of DHEPV at a dose of 300 mg/kg. The rutin concentration in the heart was 5.34 times higher than in blood plasma. Rutin penetrated mitochondria where the concentration was 1.7 times higher than in plasma and 3.18 times lower than in heart.

Previous experiments found that DHEPV limited the development of mitochondrial dysfunction *in vivo* in rats with CHF induced by administration of isoproterenol according to an increase in the respiratory coefficient of a control, the activity of the antioxidant enzyme superoxide dismutase (SOD), and a reduction in the concentration of the secondary lipid peroxidation (LPO) product malondialdehyde in mitochondria of the heart of animals that received the compound as compared to the control. DHEPV was shown to have direct antioxidant activity in various *in vitro* test systems, e.g., suppression of the chemiluminescence parameters light sum and emission amplitude, after addition of DHEPV to the test systems in various antioxidant states [13].

The results as a whole suggested that one mechanism of action of DHEPV with experimental CHF is limitation of ox-

idative stress development in mitochondria because of binding of oxygen free radicals.

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