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Determination of new derivatives of genistein in culture media by liquid chromatography

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Abstract

Methods for determination of genistein and its four new analogues in culture media have been developed to support studies on their potential anticancer activities. The investigated compounds were extracted from the media using liquid–liquid extraction with appropriate solvent. After evaporation of organic solvents each of the dry extracts was reconstituted in appropriate mobile phase. Reversed-phase HPLC was applied to quantitative determining of tested compounds. The methods are specific, sensitive and technically simple. They were used to evaluate concentration level of investigated compounds in experiments with human promyelocytic leukemia cells (HL-60 cell line).

Keywords: Genistein

1. Introduction

Genistein (**I**) (Fig. 1) is one of the most intensively studied objects among naturally occurring flavonoids, because of its common presence in soy derived foodstuff and postulated significance for chemoprevention and therapy, based on extensive data derived from epidemiological studies [1–4]. Molecular mechanisms behind its prospective medicinal applications, which include estrogenic activity, protein tyrosine kinase inhibition and topoisomerase blocking are relatively well investigated but mostly limited to in vitro experiments [5–7].

It is generally accepted that genistein demonstrates considerable anticancer potential, which stems from its influence on signal transduction, as well as from affinity towards estrogen receptors (ER) and topoisomerases. Unfortunately in order to observe therapeutically significant results, it would be necessary to attain higher intracellular concentrations of the compound, than those resulting from soy-rich diet or nutraceutical supplementation. In fact, genistein as such, is not a good drug candidate, because of extremely low solubility in both: aqueous and lipid environments, which limits its biodistribution and reduces its bioavailability. Accordingly, we have decided to explore antitumor activity of chemically modified genistein-derived compounds, with significantly increased $\log P$ values, in keeping with general principles of medicinal chemistry [8–10]. A decision of using glycosylating reagents for derivatization of **I** results from widely accepted opinion that carbohydrates are essential not only for biological recognition of endogenous compounds but also significantly influence trafficking and processing of xenobiotics [11–13]. Detailed reasoning behind structural design of these new derivatives and details of synthetic procedures by which they were prepared are described elsewhere [14].

Quantitative determination of **I**, its glycosidic precursors and their metabolites, in biological material drawn from both: native plant and animals (or humans) which fed on it, are of great significance for clinical, phytochemical and nutritional analysis. Consequently, a great deal of attention

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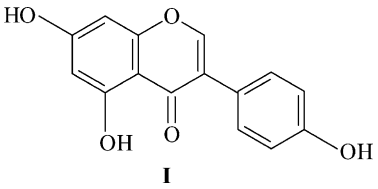


I

Fig. 1. Chemical structure of genistein.

has been committed recently to develop of reliable analytical procedures [15–17].

In contrast to the well-known flavonoid compounds present in samples of natural origin, our study has focused on four new synthetic derivatives of **I** pictured below (**II**–**V**) (Fig. 2), which are representative for a larger collection, being examined for their cytostatic and cytotoxic properties [14]. Since biodegradability was postulated in their design as a desirable feature, the question of their structural integrity in the biological media used for activity experiments has been legitimately risen.

Consequently, to support biological activity study, analytical procedure had to be elaborated for simultaneous detection and quantitative determination of genistein and its new derivatives in examined media, as well as products of their possible chemical or biological in situ transformation.

Methods of detection and determination of isoflavones have been recently reviewed in connection with their phytoestrogenic activity [15–35]. There is understandable tendency to push limits of isoflavone detection towards small fraction of nanomolar concentrations by use of sophisticated equipment. Nevertheless we decided to employ simple isocratic reversed-phase HPLC with UV detection, for analysis of isolates from the HL-60 cells culture media incubated with examined compounds (**I**–**V**) obtained after liquid–liquid extraction.

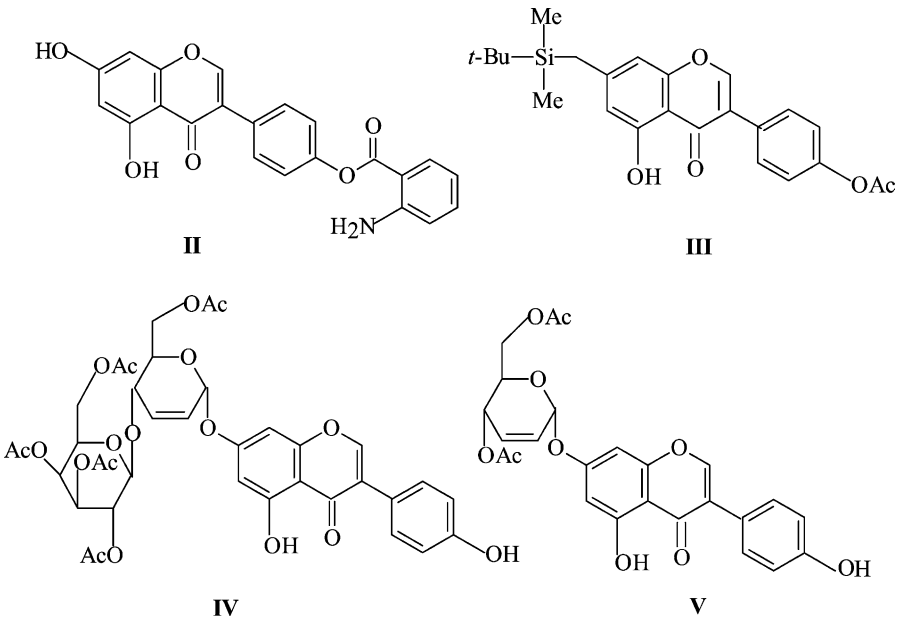
2. Experimental

2.1. Reagents and chemicals

Genistein (**I**) and its analogues (**II**–**V**) were synthesised in Pharmaceutical Research Institute (Warsaw, Poland), by methods described elsewhere [14]. Human promyelocytic leukemia (HL-60) cell line was obtained from ATCC (Manassa, VA, USA). RPMI-1640 medium was purchased from IITD (Wroclaw, Poland). Fetal calf serum and L-glutamine were obtained from Gibco Invitrogen Corporation (Grand Island, NY, USA). Non-essential amino acids and antibiotics (penicillin, streptomycin and amphotericin) were purchased from Sigma (St. Louis, MO, USA). Glucose was obtained from Pliva (Krakow, Poland). HPLC-grade acetonitryl, *n*-hexane, *n*-propanol and *tert*-butyl methyl ether (TBME) were purchased from LabScan (Dublin, Ireland), glacial acetic acid and dimethyl sulfoxide (DMSO) were from Baker (Deventer, Holland), ammonium formate was from Fluka (Busch, Switzerland), formic acid from Riedel-de Haën (Seelze, Germany). Distilled water was purified in a Millipore System Milli Q (Molsheim, France).

2.2. Culture processing

Cell culture and supernatants processing was performed in National Institute of Public Health (Warsaw, Poland) [36]. Human promyelocytic leukemia cells grew as a suspension in RPMI-1640 medium supplemented with 20% fetal calf serum, 2 mM glutamine, 1 mM non-essential amino



II **III** **IV** **V**

Fig. 2. Chemical structure of the investigated genistein analogues.

acids, 5 mg/ml glucose and antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin and 250 ng/ml amphotericin B). Cells were maintained in standard tissue culture plastic flasks 25 cm^2 at 37 °C in humidified atmosphere of 5% CO_2 in air. For experiments, exponentially growing cells were used. Cells were counted using an electronic analyzer (Coulter Particle Analyzer Z™ Series, Beckman Coulter, Germany) and suspended in fresh medium to obtain the concentration of 2.5×10^5 cell/ml. Then the cell suspension (40 ml) were plated out into 75 cm^2 culture flasks. Cells were preincubated for 1 h and then the drug solution or solvent—0.027% DMSO (drug-free suspension) was added to obtain concentration of 10 μM of each investigated compound. Cells were incubated for additional 48 h. After incubation time cell suspension were centrifuged. Supernatants (drug-free and containing the tested compound) were transferred into separate tubes and stored at −20 °C up to the moment of HPLC analysis. Experiments were performed twice.

2.3. Analytical solutions

Stock solutions were made by dissolving accurately weighed milligram quantities of the standards in DMSO to obtain concentration of 1 mg/ml. The solutions were stored at 5 °C.

Standard solutions were made by serially diluting the stock solutions with drug-free supernatant to obtain desired concentrations of the compounds. These solutions were kept at −20 °C when not in use.

2.4. Sample preparation

To 0.5 ml supernatant (containing **I**, **II**, **III**, **IV** or **V**) in 10 ml glass tube 3 ml of TBME (for **I**, **IV** and **V**) or 4 ml of *n*-hexane containing 10% of *n*-propanol (for **II**) or 4 ml mixture of *n*-hexane–*n*-propanol–acetic acid (95:5:1, v/v/v) (for **III**) were added, respectively. The solution was shaken for 10 min and centrifuged at 3500 × *g* for 10 min. After freezing at −70 °C for 15 min the organic layer was transferred to another glass tube and evaporated to dryness at 45 °C under a stream of nitrogen. The dry residue was dissolved in 200 μl an appropriate mobile phase (with vortexing for 10 s) and transferred to an autosampler vial. 50 μl aliquot was injected onto the HPLC system for analysis.

2.5. Apparatus

Liquid chromatography was performed with HPLC system which consisted of an isocratic pump (Model LC-6A), a UV-Vis detector (Model SPD-6A) and an autosampler (Model SIL-9A) with 50 μl sample loop (Shimadzu Europa, Duisburg, Germany). Chromatographic data were collected and processed with software Chromax 2001 ver. 1.5 (PolLab).

2.6. Chromatographic conditions

The chromatographic separation was performed using, respectively,

- Symmetry C18 (25 cm × 4.6 mm i.d.) 5 μm (Waters) column preceded by C18 guard column (4 mm × 3 mm i.d.) (Phenomenex)—for determination **I**.
- LC8 (15 cm × 4.6 mm i.d.) 5 μm (Supelco) column preceded by C8 guard column (4 mm × 3 mm i.d.) (Phenomenex)—for determinations **II** and **III**.
- LC18 (15 cm × 4.6 mm i.d.) 3 μm (Supelco) column preceded by C18 guard column (4 mm × 3 mm i.d.) (Phenomenex)—for determination **IV**.
- Luna C18 (25 cm × 4.6 mm i.d.) 5 μm (Phenomenex) column preceded by C18 guard column (4 mm × 3 mm i.d.) (Phenomenex)—for determination **V**.

The mobile phases were as follows:

I: mixture of acetonitrile and 0.1 M ammonium formate (30:70, v/v). Flow rate: 1 ml/min.
II: mixture of acetonitrile and 0.05 M ammonium formate buffer pH 4.0 (47:53, v/v). Flow rate: 1.5 ml/min.
III: mixture of acetonitrile and water (65:35, v/v). Flow rate: 2 ml/min.
IV: mixture of acetonitrile and 0.1 M ammonium formate (50:50, v/v). Flow rate: 1 ml/min.
V: mixture of acetonitrile and 0.1 M ammonium formate (55:45, v/v). Flow rate: 1 ml/min.

The phases were filtered using 0.45 μm Nylon 66 membrane (Supelco) and ultrasonically degassed prior to use. HPLC analyses were performed at ambient temperature with ultraviolet detection (262 nm). A 50 μl portion of the samples was injected on appropriate HPLC column.

2.7. LOD and LOQ

The limit of detection (LOD) was defined as the sample concentration of a tested compound resulting in peak height of thrice the noise level.

The limit of quantitation (LOQ) was the lowest point on the calibration curve which can be detected with variation below 15%. The results are shown in Table 1.

2.8. Calibration

Calibration was performed by serial dilution of known amount of the investigated compounds in a drug free supernatant within an appropriate range depending on an expected concentration of an investigated compound. The standards were extracted according to the procedures described in Section 2.3. Standard curves were constructed by plotting the peak-area (for **I**) or peak-height (for **II**–**IV**) against the concentration of the respective compound (Figs. 3–6). Since compound **V** was not found in the tested

Table 1
LOD and LOQ values for the investigated compounds

Compound	LOD (μM)	LOQ (μM)
I[a]	0.0037	0.0370
II[b]	0.0026	0.0128
III[c]	0.0047	0.0230
IV[d]	0.0052	0.0130
V[e]	0.0008	0.0021

[a] Chromatographic conditions—column: Symmetry C18 (25 cm × 4.6 mm i.d.) 5 μm (Waters); guard column: C18 (4 mm × 3 mm i.d.) (Phenomenex); mobile phase: mixture of acetonitrile and 0.1 M ammonium formate (30:70, v/v); flow rate: 1 ml/min; temperature: ambient; detection: 262 nm. Loop: 50 μl.

[b] Chromatographic conditions—column: LC8 (15 cm × 4.6 mm i.d.) 5 μm (Supelco); guard column: C8 (4 mm × 3 mm i.d.) (Phenomenex); mobile phase: mixture of acetonitrile and 0.05 M ammonium formate buffer pH 4 (47:53, v/v); flow rate: 1.5 ml/min; temperature: ambient; detection: 262 nm. Loop: 50 μl.

[c] Chromatographic conditions: column: LC8 (15 cm × 4.6 mm i.d.) 5 μm (Supelco); guard column: C8 (4 mm × 3 mm i.d.) (Phenomenex); mobile phase: mixture of acetonitrile and water (65:35, v/v); flow rate: 2 ml/min; temperature: ambient; detection: 262 nm. Loop: 50 μl.

[d] Chromatographic conditions: column: LC18 (15 cm × 4.6 mm i.d.) 3 μm (Supelco); guard column: C18 (4 mm × 3 mm i.d.) (Phenomenex); mobile phase: mixture of acetonitrile and 0.1 M ammonium formate (50:50, v/v); flow rate: 1 ml/min; temperature: ambient; detection: 262 nm. Loop: 50 μl.

[e] Chromatographic conditions—column: Luna C18 (25 cm × 4.6 mm i.d.) 5 μm (Phenomenex); guard column: C18 (4 mm × 3 mm i.d.) (Phenomenex); mobile phase: mixture of acetonitrile and 0.1 M ammonium formate (55:45, v/v); flow rate: 1 ml/min; temperature: ambient; detection: 262 nm. Loop: 50 μl.

supernatants, it was no reason to prepare any calibration curve for its quantitative determination. Linear least squares regression was applied to determine the slopes, intercepts and correlation coefficients of the calibration curves. To construct a calibration curve at least six concentrations of a standard were taken. The lowest concentration of the standard within each calibration curve was LOQ value.

Table 2
Recovery of the investigated compounds from their supernatants

Compound	Amount of the tested compound added to blank supernatant (μM)	Recovery (%),[a] mean ± S.D.
I	1.85	96.53 ± 1.89[b]
I	5.56	96.45 ± 1.86[b]
I	11.11	96.03 ± 0.64[b]
II	0.05	92.66 ± 3.78[c]
III	0.12	75.26 ± 3.84[d]
IV	0.65	85.67 ± 3.38[e]
V	0.02	77.87 ± 3.19[f]

[a] Tabulated values are the mean at least of three replicate determinations.

[b] Chromatographic conditions—column: Symmetry C18 (25 cm × 4.6 mm i.d.) 5 μm (Waters); guard column: C18 (4 mm × 3 mm i.d.) (Phenomenex); mobile phase: mixture of acetonitrile and 0.1 M ammonium formate (30:70, v/v); flow rate: 1 ml/min; temperature: ambient; detection: 262 nm. Loop: 50 μl.

[c] Chromatographic conditions—column: LC8 (15 cm × 4.6 mm i.d.) 5 μm (Supelco); guard column: C8 (4 mm × 3 mm i.d.) (Phenomenex); mobile phase: mixture of acetonitrile and 0.05 M ammonium formate buffer pH 4 (47:53, v/v); flow rate: 1.5 ml/min; temperature: ambient; detection: 262 nm. Loop: 50 μl.

[d] Chromatographic conditions—column: LC8 (15 cm × 4.6 mm i.d.) 5 μm (Supelco); guard column: C8 (4 mm × 3 mm i.d.) (Phenomenex); mobile phase: mixture of acetonitrile and water (65:35, v/v); flow rate: 2 ml/min; temperature: ambient; detection: 262 nm. Loop: 50 μl.

[e] Chromatographic conditions—column: LC18 (15 cm × 4.6 mm i.d.) 3 μm (Supelco); guard column: C18 (4 mm × 3 mm i.d.) (Phenomenex); mobile phase: mixture of acetonitrile and 0.1 M ammonium formate (50:50, v/v); flow rate: 1 ml/min; temperature: ambient; detection: 262 nm. Loop: 50 μl.

[f] Chromatographic conditions—column: Luna C18 (25 cm × 4.6 mm i.d.) 5 μm (Phenomenex); guard column: C18 (4 mm × 3 mm i.d.) (Phenomenex); mobile phase: mixture of acetonitrile and 0.1 M ammonium formate (55:45, v/v); flow rate: 1 ml/min; temperature: ambient; detection: 262 nm. Loop: 50 μl.

2.9. Recovery

The recovery of each of the investigated compounds from the appropriate supernatant was calculated by comparing

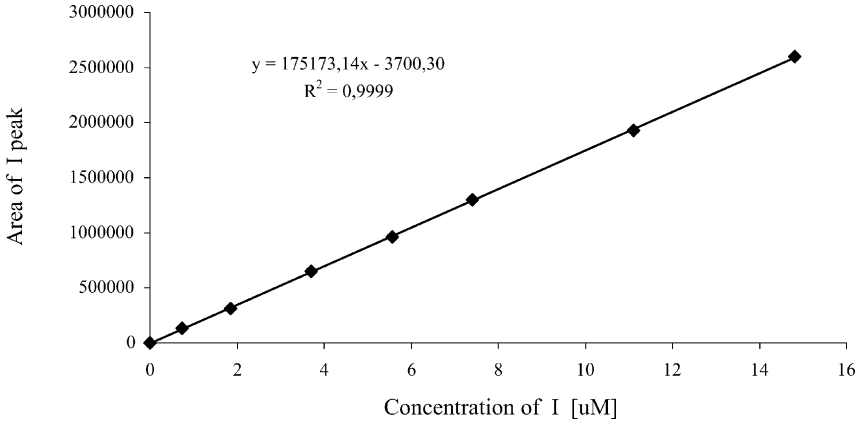


Fig. 3. Calibration curve for **I**. Chromatographic conditions—column: Symmetry C18 (25 cm × 4.6 mm i.d.) 5 μm (Waters); guard column: C18 (4 mm × 3 mm i.d.) (Phenomenex); mobile phase: mixture of acetonitrile and 0.1 M ammonium formate (30:70, v/v); flow rate: 1 ml/min; temperature: ambient; detection: 262 nm. Loop: 50 μl.

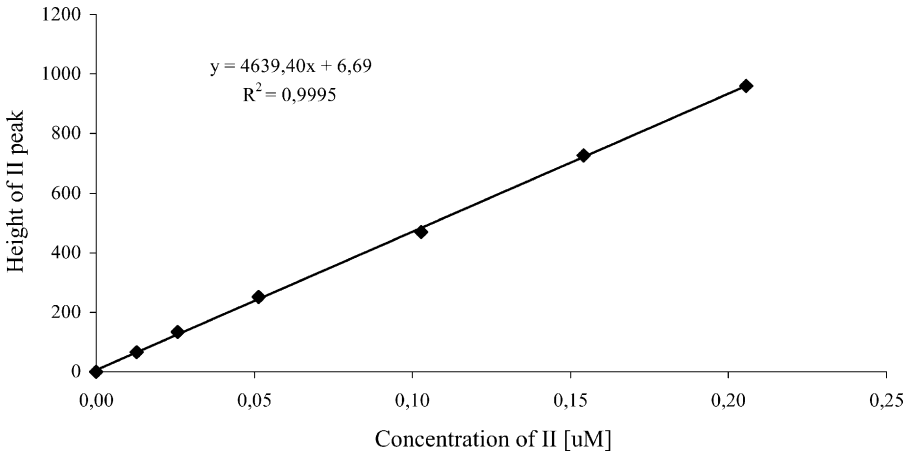


Fig. 4. Calibration curve for **II**. Chromatographic conditions—column: LC8 (15 cm × 4.6 mm i.d.) 5 μm (Supelco); guard column: C8 (4 mm × 3 mm i.d.) (Phenomenex); mobile phase: mixture of acetonitrile and 0.05 M ammonium formate buffer pH 4 (47:53, v/v); flow rate: 1.5 ml/min; temperature: ambient; detection: 262 nm. Loop: 50 μl.

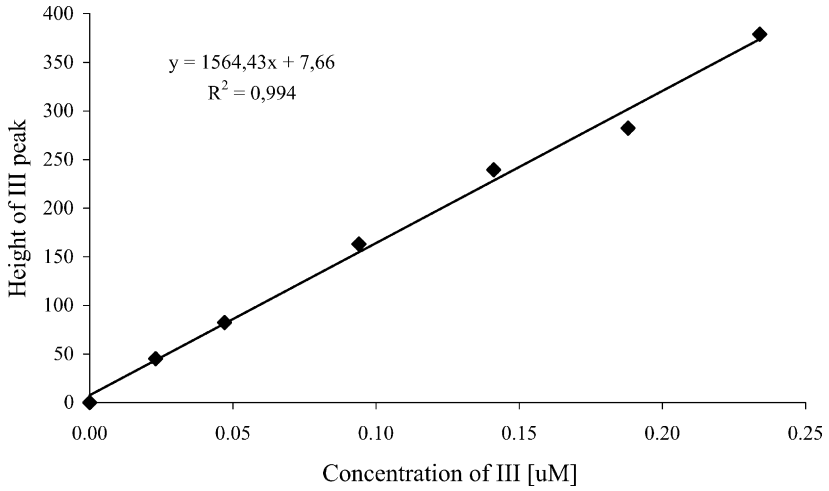


Fig. 5. Calibration curve for **III**. Chromatographic conditions—column: LC8 (15 cm × 4.6 mm i.d.) 5 μm (Supelco); guard column: C8 (4 mm × 3 mm i.d.) (Phenomenex); mobile phase: mixture of acetonitrile and water (65:35, v/v); flow rate: 2 ml/min; temperature: ambient; detection: 262 nm. Loop: 50 μl.

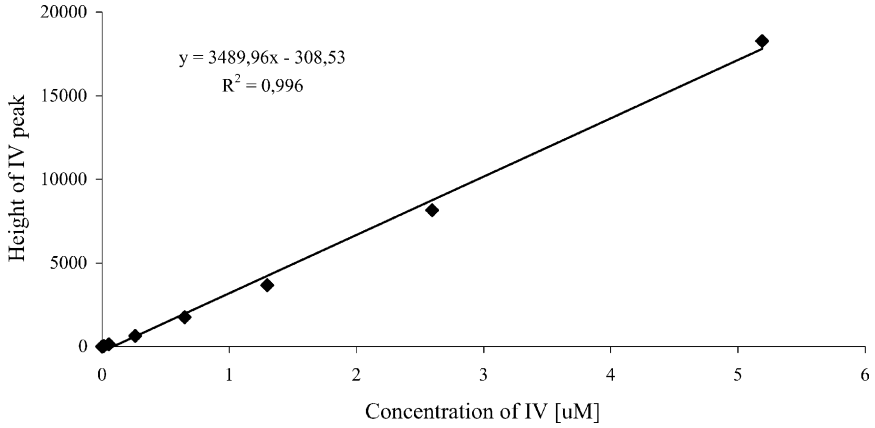


Fig. 6. Calibration curve for **IV**. Chromatographic conditions—column: LC18 (15 cm × 4.6 mm i.d.) 3 μm (Waters); guard column: C18 (4 mm × 3 mm i.d.) (Phenomenex); mobile phase: mixture of acetonitrile and 0.1 M ammonium formate (50:50, v/v); flow rate: 1 ml/min; temperature: ambient; detection: 262 nm. Loop: 50 μl.

the peak-area (for **I**) or peak-height (for other compounds) of known concentration of the standard in adequate mobile phase (representing 100% recovery) and the same amount of the standard added to drug-free supernatant and recovered after extraction. Assay were performed on three levels of the concentration within calibration range (for **I**) or only on one level, approached to expected in a supernatant (for **II**–**V**). Each concentration level was prepared in at least three samples. The results are collected in Table 2.

3. Results and discussion

In the search for effective way to prepare HPLC samples of investigated compounds from their supernatants we chose the liquid–liquid extraction technique for its speed and simplicity. Supko et al. [32] have successfully applied *tert*-butyl methyl ether (TBME) for extraction of genistein (**I**) from plasma. We have adopted this procedure for extraction of compounds **I**, **IV** and **V** from processed cell cultures (supernatants) using considerably reduced organic solvent-to-supernatant ratio. Observed recovery: 96.3, 85.7 and 77.9% for **I**, **IV** and **V**, respectively (Table 2), was considered satisfactory to perform analyses.

Initial solvent screening for extraction of **II** and **III** included: TBME, ethyl acetate, ethyl ether, dichloromethane and hexane. Eventually, it was found that a mixture of *n*-hexane–*n*-propanol (9:1; v/v) and mixture of *n*-hexane–*n*-propanol–acetic acid (95:5:1; v/v/v) gave sufficient recovery values for **II** and **III** (92.7 and 75.3%, respectively, Table 2).

To determine of our compounds after extraction from their supernatants by HPLC we checked several chromatographic columns with different stationary phases (CN, C8 and C18) and variety of mobile phases (mixtures of phosphate buffers or ammonium formate or ammonium acetate pH 3–7 with methanol or acetonitrile as organic modifiers). Generally, good separations were obtained on C18 columns when ammonium formate and acetonitrile were components of mobile phases. However for more hydrophobic compounds **II** and **III** C8 stationary phase can be recommended to limit their retention time.

In the case of **I**, we have tried to use C8 column according to Supko et al. [32]. Problems with separation of the main peak for some substances co-extracted with **I** were encountered. These difficulties could be overcome by use of 25 cm C18 Symmetry column from Waters. Mean value of retention time for **I** was estimated as 16.27 ± 0.52 min ($n = 23$).

It was demonstrated that chromatograms of the drug-free supernatants did not show any interfering compounds extracted from the samples (Figs. 7A, 8A, 9A, 10A and 11A). Therefore, the methods for determination of the investigated compounds are sufficiently selective.

Typical chromatograms of drug-free supernatants spiked with appropriate compounds are presented in Figs. 7B, 8B, 9B, 10B and 11B. Mean values of retention time for **II** was calculated as 7.91 ± 0.34 min ($n = 32$), for **III** 8.82 ± 0.05 min ($n = 21$), for **IV** 9.89 ± 0.45 min ($n = 31$) and for **V** 12.28 ± 0.49 ($n = 22$).

The chromatograms of the supernatants containing the respective compounds are shown in Figs. 7C, 8C, 9C, 10C and 11C.

Calibration graphs were prepared within the following ranges:

- for **I**: 0.74–14.81 μM (Fig. 3);
- for **II**: 0.01–0.21 μM (Fig. 4);
- for **III**: 0.02–0.23 μM (Fig. 5);
- for **IV**: 0.01–5.19 μM (Fig. 6).

Since compound **V** was not found in the tested supernatants, the respective calibration curve was not constructed. A good correlation was observed between peak-height ratios (for **I**) or peak-area (for other compound) ratios and concentrations of the analysed compounds. From least-squares regression analysis resulted the following regression lines:

- for **I**: $y = 17\,513.14x - 370\,030$, $R^2 = 1.000$;
- for **II**: $y = 4639.40x - 6.69$, $R^2 = 0.999$;
- for **III**: $y = 1564.43x - 7.66$, $R^2 = 0.994$;
- for **IV**: $y = 3489.96x - 308.53$, $R^2 = 0.996$,

where x is the concentration of an investigated compound in the respective supernatant, y the peak-height (**I**) or peak-area (**II**, **III**, **IV** and **V**) obtained from a chromatogram, R the correlation coefficient.

Minimum detectable concentrations (LOD) and quantitative limits (LOQ) of the investigated compounds are shown in Table 1. The methods for determination of the investigated compounds are properly sensitive. For **I:** the LOD and LOQ values were estimated as 0.0037 and 0.0370 μM, respectively; for **II**: 0.0026 and 0.0128 μM, for **III**: 0.0047 and 0.0230 μM, for **IV**: 0.0052 and 0.0130 μM and for **V**: 0.0008 and 0.0021 μM.

The tested compounds could have been biodegraded in culture media to genistein (**I**), its concentration in supernatants was also analysed. Chromatograms of the supernatants containing the appropriate compounds obtained under the conditions elaborated for **I** are shown in Figs. 12–15, respectively.

The results of the assayed supernatants (from two independent culture media experiments) with each of the five compounds are summarised in Table 3.

The results show, that entire amount of the **I** added to the culture medium (10 μM) was determined after extraction from supernatant (100%). It proves that **I** is completely stable during incubation with HL-60 cells (Fig. 7C). The retention time of **I**: 16.70 min.

In the case of compound **II** the result is dramatically different. In the supernatant **II** after extraction we found only 0.02 μM (0.2%) of the tested compound and near 10 μM of **I**. Apparently, stability of **II** during the incubation is very low, which can be easily justified by the presence of weak amino acid–phenol ester bond. Retention time of **II**: 8.14 min (Fig. 8C), retention time of **I**: 17.19 min (Fig. 12).

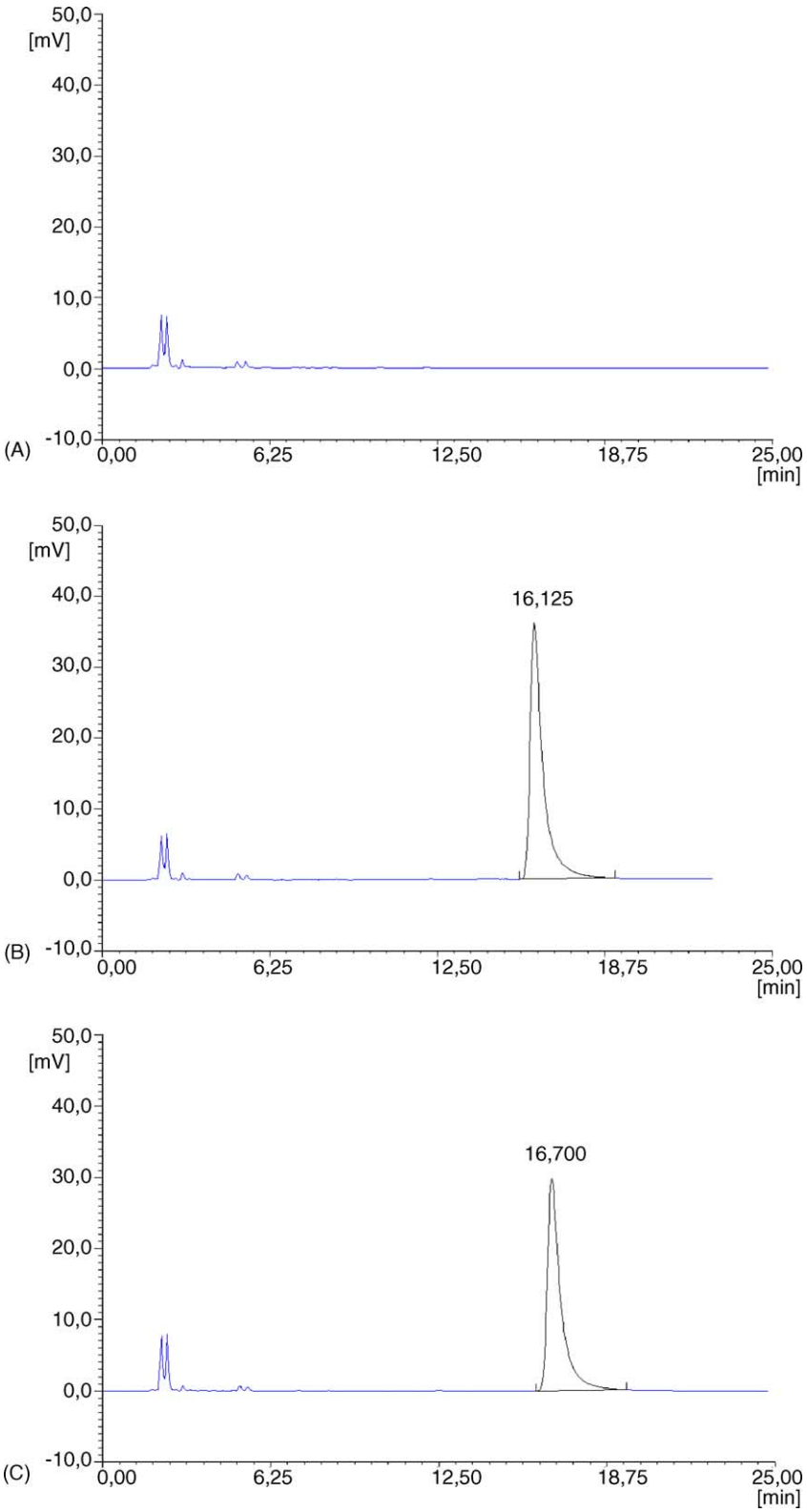


Fig. 7. Chromatograms of drug-free supernatant (A), drug-free supernatant spiked with 5.58 μM of **I**: retention time of genistein peak 16.13 min (B) and supernatant **I**: retention time of genistein peak 16.70 min. (C). Chromatographic conditions—column: Symmetry C18 (25 cm × 4.6 mm i.d.) 5 μm (Waters); guard column: C18 (4 mm × 3 mm i.d.) (Phenomenex); mobile phase: mixture of acetonitrile and 0.1 M ammonium formate (30:70, v/v); flow rate: 1 ml/min; temperature: ambient; detection: 262 nm. Loop: 50 μl.

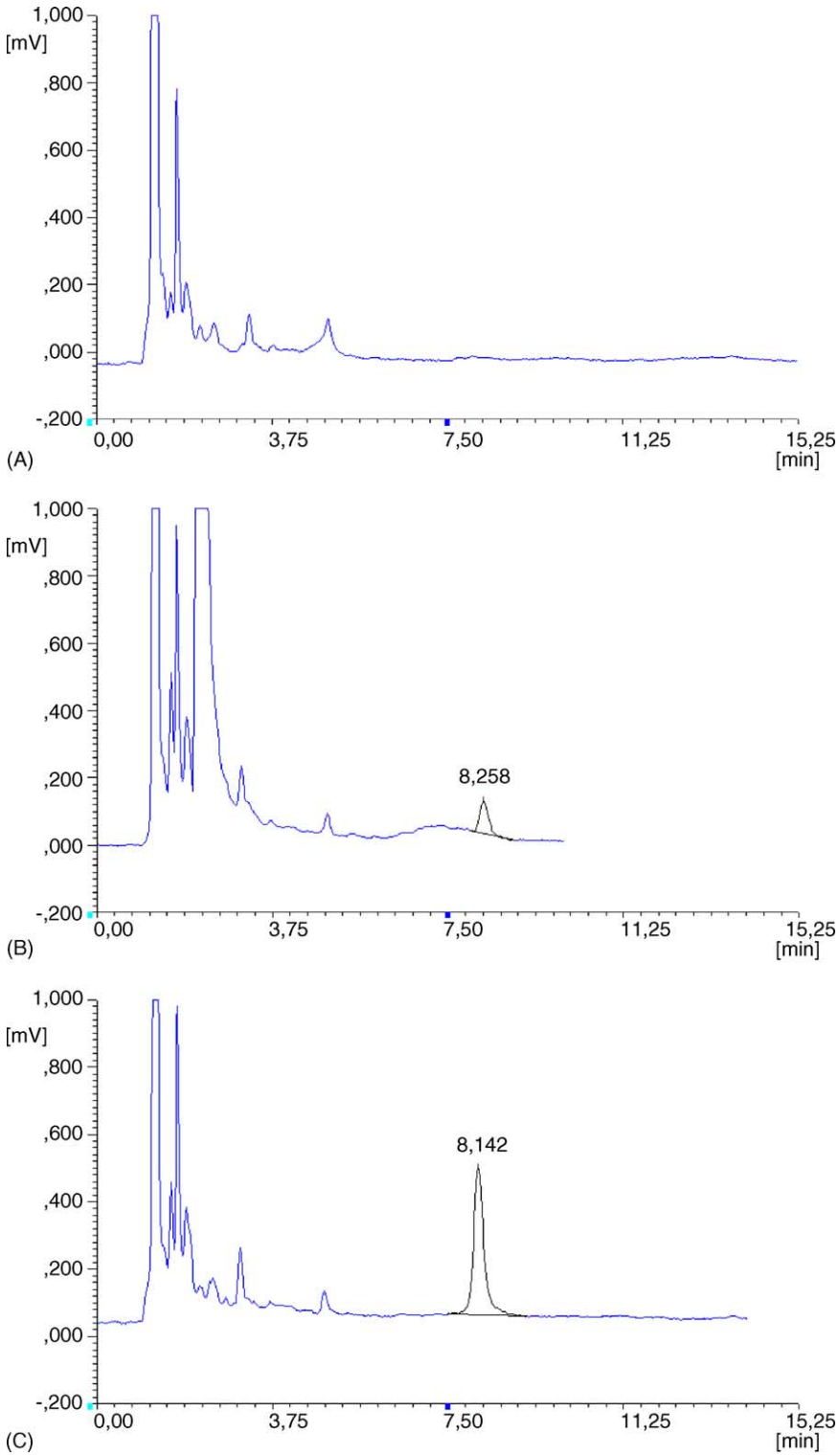


Fig. 8. Chromatograms of drug-free supernatant (A), dru-free supernatant spiked with 0.05 μM of **II**: retention time of **II** peak 8.26 min (B) and supernatant **II**: retention time of **II** peak 8.14 min. (C). Chromatographic conditions—column: LC8 (15 cm × 4.6 mm i.d.) 5 μm (Supelco); guard column: C8 (4 mm × 3 mm i.d.) (Phenomenex); mobile phase: mixture of acetonitrile and 0.05 M ammonium formate buffer pH 4 (47:53, v/v); flow rate: 1.5 ml/min; temperature: ambient; detection: 262 nm. Loop: 50 μl.

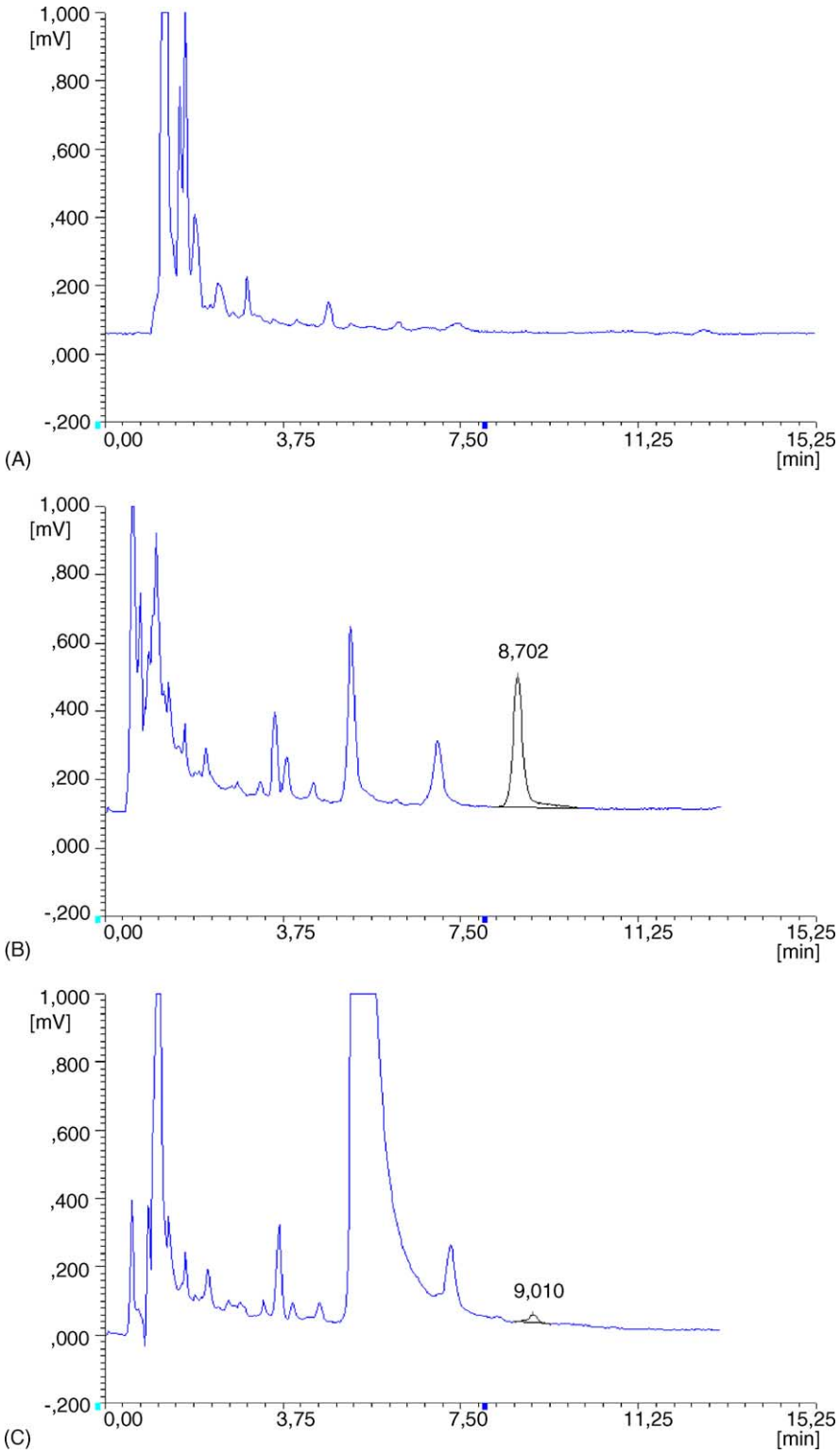


Fig. 9. Chromatograms of drug-free supernatant (A), drug-free supernatant spiked with 0.12 μM of **III**: retention time of **III** peak 8.70 min (B) and supernatant **III**: retention time of **III** peak 9.01 min. (C) Chromatographic conditions—column: LC8 (15 cm × 4.6 mm i.d.) 5 μm (Supelco); guard column: C8 (4 mm × 3 mm i.d.) (Phenomenex); mobile phase: mixture of acetonitrile and water (65:35, v/v); flow rate: 2 ml/min; temperature: ambient; detection: 262 nm. Loop: 50 μl.

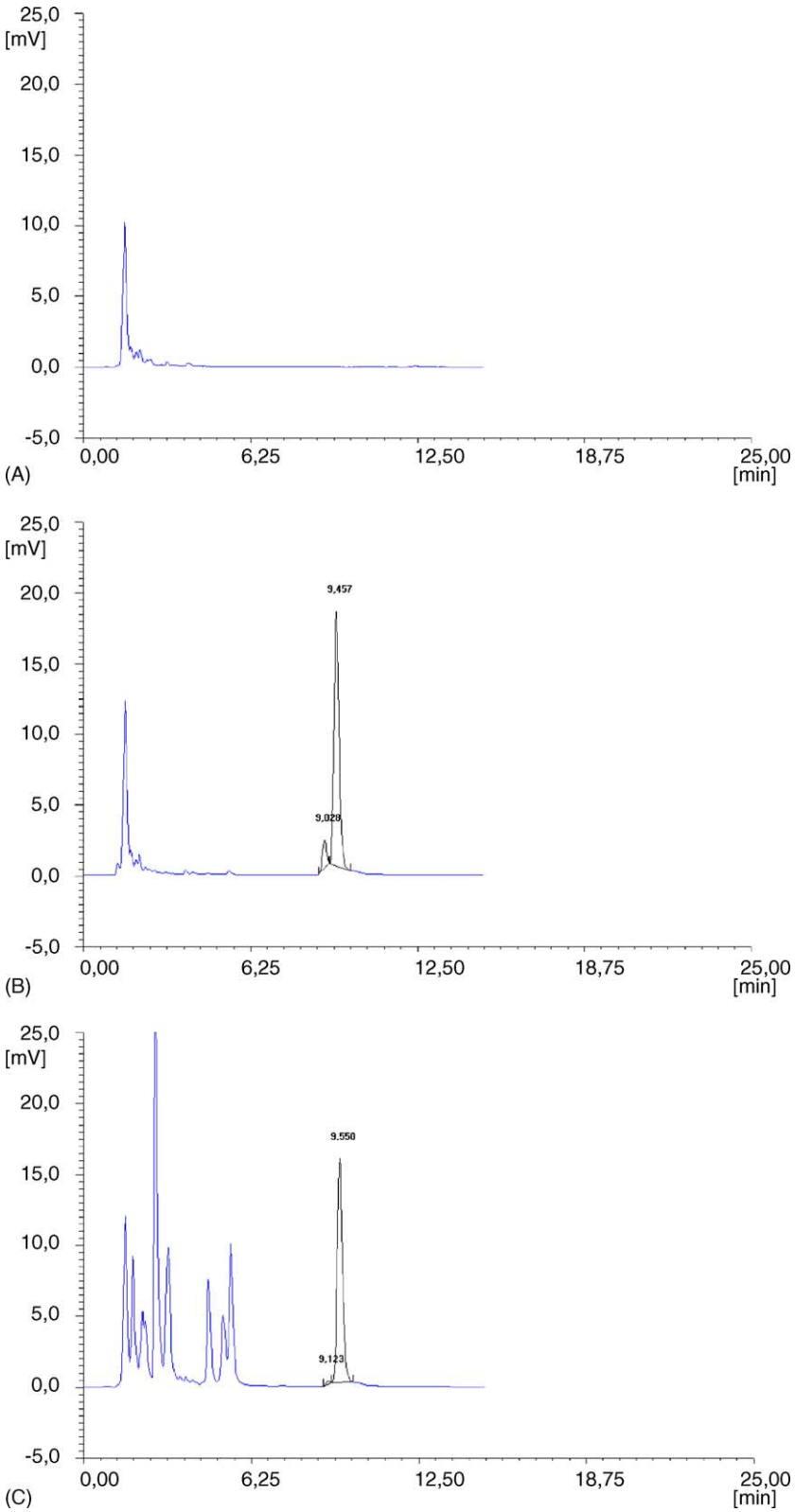


Fig. 10. Chromatograms of drug-free supernatant (A), drug-free supernatant spiked with 2.60 μM of **IV**: retention time of **IV** peak 9.46 min (B) and supernatant **IV**: retention time of **IV** peak 9.55 min. (C) Chromatographic conditions—column: LC18 (15 cm × 4.6 mm i.d.) 3 μm (Supelco); guard column: C18 (4 mm × 3 mm i.d.) (Phenomenex); mobile phase: mixture of acetonitrile and 0.1 M ammonium formate (50:50, v/v); flow rate: 1 ml/min; temperature: ambient; detection: 262 nm. Loop: 50 μl.

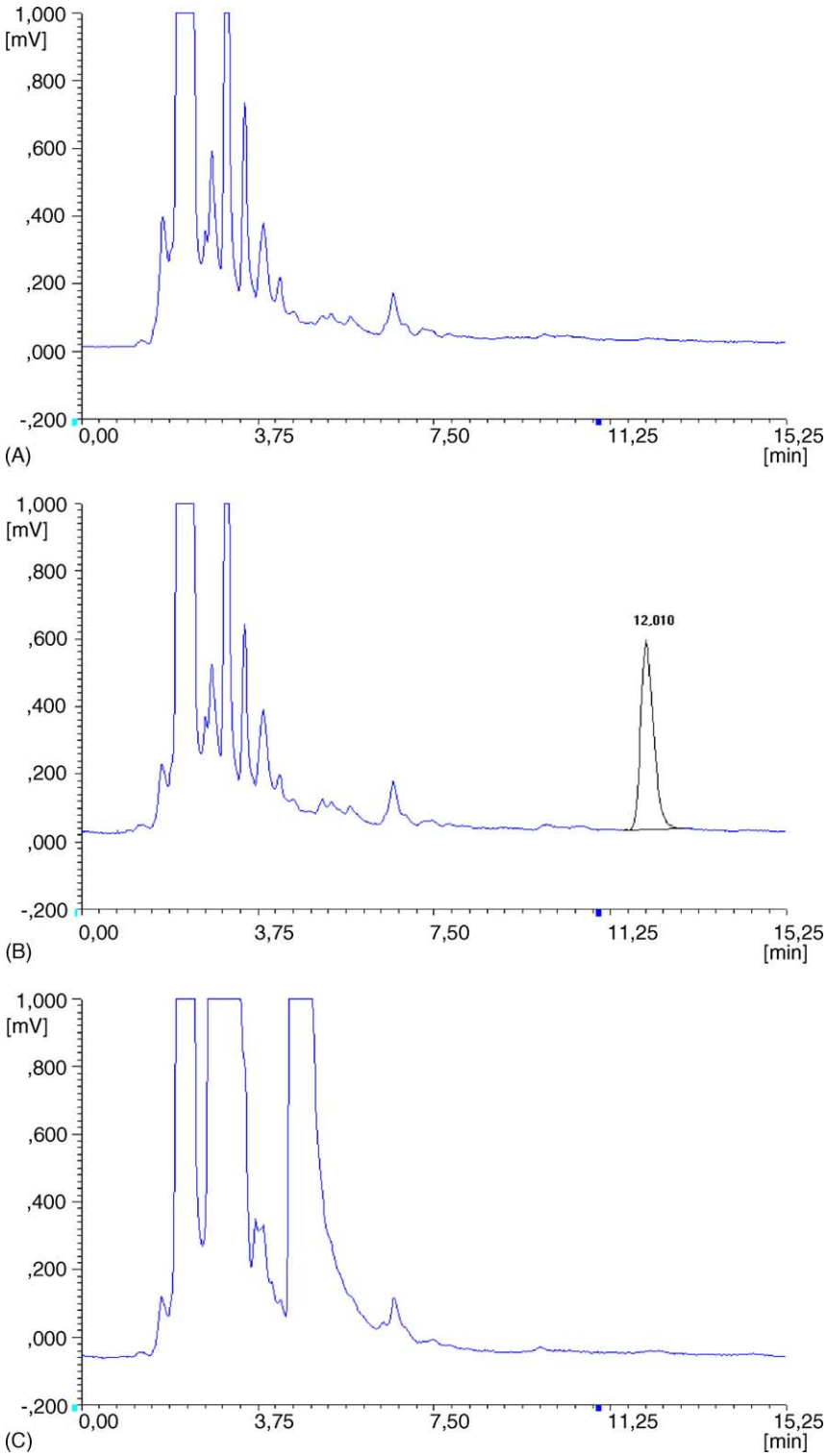


Fig. 11. Chromatograms of drug-free supernatant (A), drug-free supernatant spiked with 0.04 μM of **V**: retention time of **V** peak 12.01 min (B) and supernatant **V**: lack of peak in the retention time of **V** (C) Chromatographic conditions—column: Luna C18 (25 cm × 4.6 mm i.d.) 5 μm (Phenomenex); guard column: C18 (4 mm × 3 mm i.d.) (Phenomenex); mobile phase: mixture of acetonitrile and 0.1 M ammonium formate (55:45, v/v); flow rate: 1 ml/min; temperature: ambient; detection: 262 nm. Loop: 50 μl.

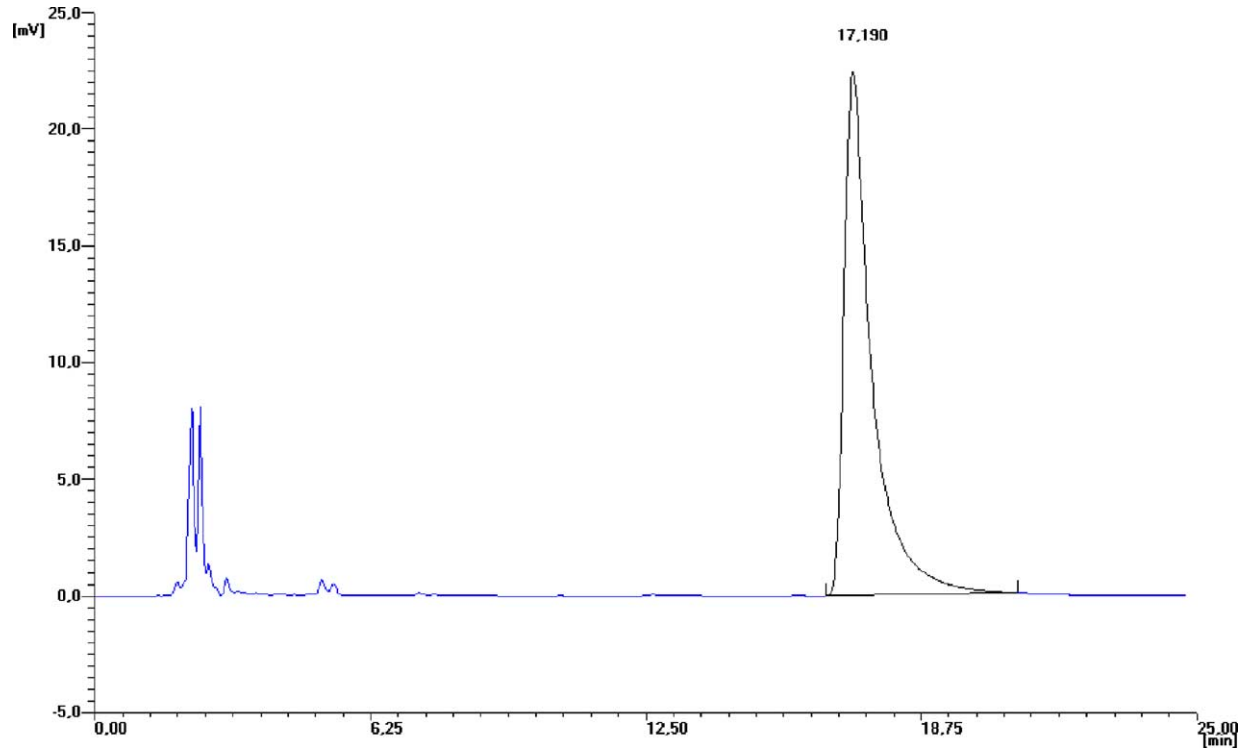


Fig. 12. Chromatogram of Supernatant **II**. Main peak of genistein (retention time 17.19 min). Chromatographic conditions—column: Symmetry C18 (25 cm × 4.6 mm i.d.) 5 μm (Waters); guard column: C18 (4 mm × 3 mm i.d.) (Phenomenex); mobile phase: mixture of acetonitrile and 0.1 M ammonium formate (30:70, v/v); flow rate: 1 ml/min; temperature: ambient; detection: 262 nm. Loop: 50 μl.

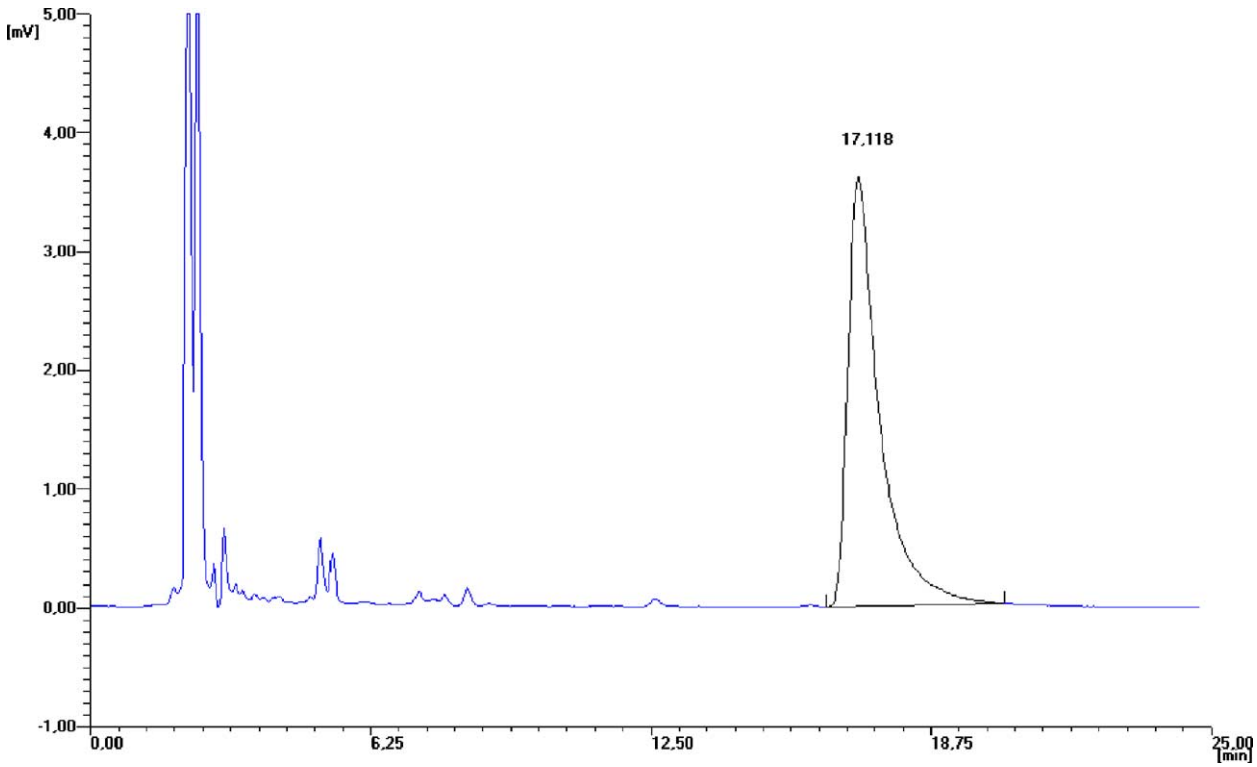


Fig. 13. Chromatogram of Supernatant **III**. Main peak of genistein (retention time 17.12 min). Chromatographic conditions—column: Symmetry C18 (25 cm × 4.6 mm i.d.) 5 μm (Waters); guard column: C18 (4 mm × 3 mm i.d.) (Phenomenex); mobile phase: mixture of acetonitrile and 0.1 M ammonium formate (30:70, v/v); flow rate: 1 ml/min; temperature: ambient; detection: 262 nm. Loop: 50 μl.

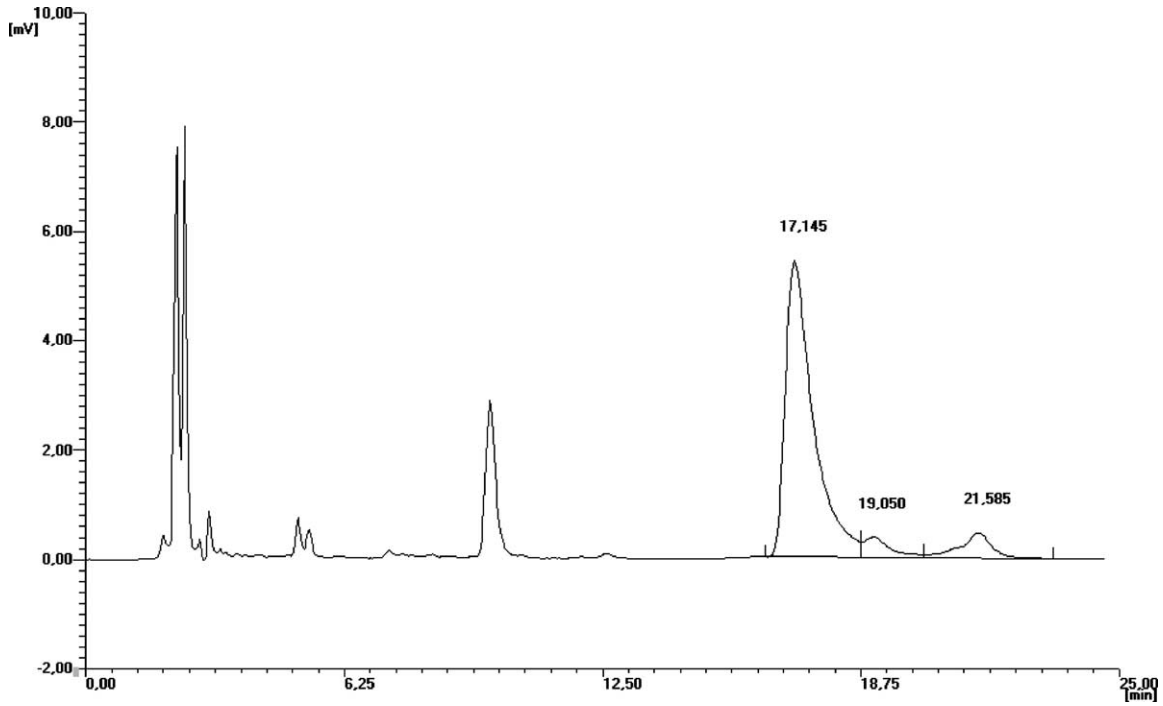


Fig. 14. Chromatogram of Supernatant **IV**. Main peak of genistein (retention time 17.15 min). Chromatographic conditions—column: Symmetry C18 (25 cm × 4.6 mm i.d.) 5 μm (Waters); guard column: C18 (4 mm × 3 mm i.d.) (Phenomenex); mobile phase: mixture of acetonitrile and 0.1 M ammonium formate (30:70, v/v); flow rate: 1 ml/min; temperature: ambient; detection: 262 nm. Loop: 50 μl.

The results obtained in our investigation on supernatant **III** indicated that unmodified compound **III** was found in about 0.07 μM (7%) while **I** was found in about 1.7 μM (17%). Additionally we have found in the tested supernatant a new compound, different from the above, which was considered to be a metabolite of **III**. We hypothesize that biocatalytic deacetylation takes place as the first step of conversion of **III**–**I**. Retention time of **III**: 9.01 min (Fig. 9C), retention time of **I**: 17.12 min (Fig. 13).

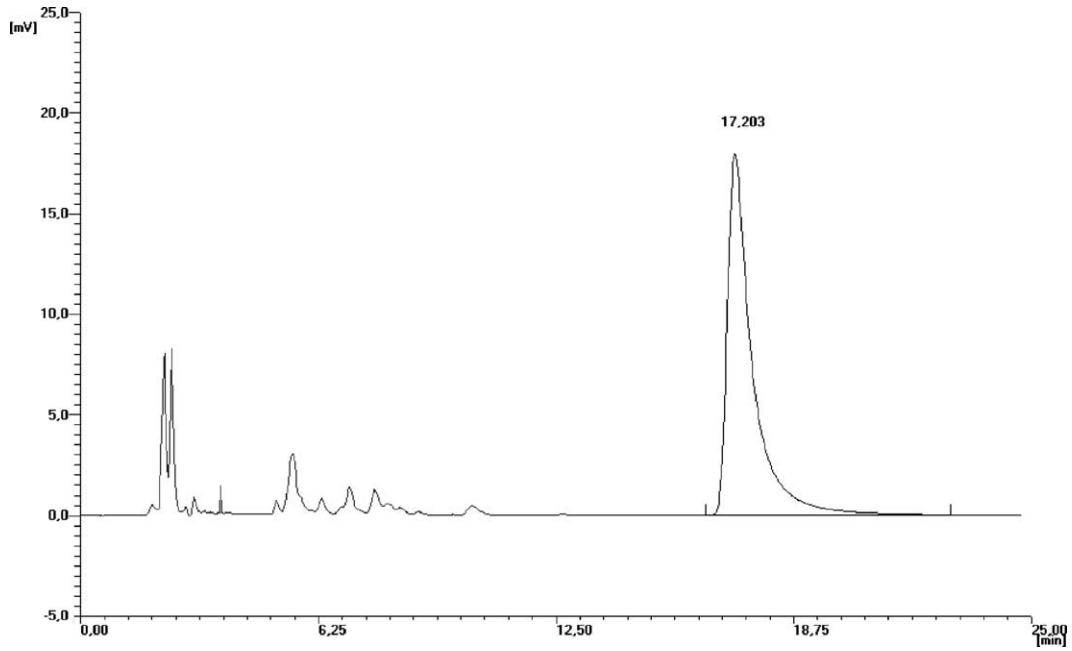


Fig. 15. Chromatogram of Supernatant **V**. Main peak of genistein (retention time 17.20 min). Chromatographic conditions—column: Symmetry C18 (25 cm × 4.6 mm i.d.) 5 μm (Waters); guard column: C18 (4 mm × 3 mm i.d.) (Phenomenex); mobile phase: mixture of acetonitrile and 0.1 M ammonium formate (30:70, v/v); flow rate: 1 ml/min; temperature: ambient; detection: 262 nm. Loop: 50 μl.

Table 3
Content of genistein and its derivatives in the tested supernatants

Compound	Amount of the compound added to culture medium (μM)	Amount[a] of the compound determined in tested supernatant (μM)	Amount[a] of genistein determined in tested supernatant (μM)
I	10	–	10.34[c]
II	10	0.02[d]	10.05[c]
III	10	0.07[b,e]	1.68[c]
IV	10	4.99[f]	2.57[c]
V	10	<LOD[g]	8.10[c]

[a] Mean of two values obtained from independently prepared supernatants.
[b] There was observed considerable amount of a metabolite in the tested supernatants.
[c] Chromatographic conditions—column: Symmetry C18 (25 cm × 4.6 mm i.d.) 5 μm (Waters); guard column: C18 (4 mm × 3 mm i.d.) (Phenomenex); mobile phase: mixture of acetonitrile and 0.1 M ammonium formate (30:70, v/v); flow rate: 1 ml/min; temperature: ambient; detection: 262 nm. Loop: 50 μl.
[d] Chromatographic conditions—column: LC8 (15 cm × 4.6 mm i.d.) 5 μm (Supelco); guard column: C8 (4 mm × 3 mm i.d.) (Phenomenex); mobile phase: mixture of acetonitrile and 0.05 M ammonium formate buffer pH 4 (47:53, v/v); flow rate: 1.5 ml/min; temperature: ambient; detection: 262 nm. Loop: 50 μl.
[e] Chromatographic conditions—column: LC8 (15 cm × 4.6 mm i.d.) 5 μm (Supelco); guard column: C8 (4 mm × 3 mm i.d.) (Phenomenex); mobile phase: mixture of acetonitrile and water (65:35, v/v); flow rate: 2 ml/min; temperature: ambient; detection: 262 nm. Loop: 50 μl.
[f] Chromatographic conditions—column: LC18 (15 cm × 4.6 mm i.d.) 3 μm (Supelco); guard column: C18 (4 mm × 3 mm i.d.) (Phenomenex); mobile phase: mixture of acetonitrile and 0.1 M ammonium formate (50:50, v/v); flow rate: 1 ml/min; temperature: ambient; detection: 262 nm. Loop: 50 μl.
[g] Chromatographic conditions—column: Luna C18 (25 cm × 4.6 mm i.d.) 5 μm (Phenomenex); guard column: C18 (4 mm × 3 mm i.d.) (Phenomenex); mobile phase: mixture of acetonitrile and 0.1 M ammonium formate (55:45, v/v); flow rate: 1 ml/min; temperature: ambient; detection: 262 nm. Loop: 50 μl.

In the case of compound **IV** we found about 50% of unmodified **IV** and about 25% of **I** in supernatant **IV**. Retention time of **IV**: 9.55 min (Fig. 10C), retention time of **I**: 17.15 min (Fig. 14; Table 3).

Compound **V** was not detected at all in the corresponding supernatant (Fig. 11C), while parent genistein (**I**) was determined at the level about 80% of the initial concentration. Retention time of **I**: 17.20 min (Fig. 15).

To sum up, our HPLC analyses allowed us to shed some light on the metabolic fate of genistein (**I**) and its synthetic derivatives under cell culture conditions. First of all we have confirmed that genistein is a stable compound that does not undergo metabolism or decomposition in in vitro system. The conclusion is in good agreement with the results obtained by other authors [37]. Furthermore we were able to prove that some new genistein derivatives liberate the parental isoflavone when incubated with cancer cells. This finding confirmed of our initial assumptions and expectations. However the rate of the observed biodegradation process should be termed as very slow and the compounds as relatively stable since after 48 h of incubation with intensively metabolizing cells, at elevated temperature (37 °C) and in the presence of a number of serum enzymes only 17, 25 and 80% of compound **III**, **IV** and **V**, respectively, underwent complete conversion to free genistein. The only exception was genistein ester with anthranilic acid (compound **II**), which was completely hydrolyzed to the parent isoflavone. However in this case it is only possible to conclude that the hydrolysis took less than 48 h but the exact rate of this process can not be precisely defined. We can expect this process is not very rapid since despite complete hydrolysis, it showed different mechanism of action against cancer cells comparing to genistein [14]. On the whole, presented above considerations generally support the result of our biological studies, where we found new derivatives to act in a different way than genistein does. At the same time biological studies along with HPLC findings strongly suggest that derivatives do not undergo rapid bioconversion to free genistein either in culture medium or inside cancer cells.

In conclusion, efficient extraction method provides solid foundation for simple and sensitive procedures based on isocratic HPLC with UV detector, which can be applied for detection and determination of genistein and its derivatives in cell line culture medium and similar biological materials. Elaborated method can provide an important and useful information that may help to design new biologically active compounds and to follow their metabolic fates.

Acknowledgements

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