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Radioimmunoassay of Free Genistein in Human Serum

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Two radioimmunoassay (RIA) systems for genistein have been established, based on polyclonal antibodies against genistein-4′-*O*-(carboxymethyl)ether-bovine serum albumin and genistein-7-*O*-(carboxymethyl)ether-bovine serum albumin conjugates. The sensitivities of assays were 4.44 and 10.4 fmol (1.2 and 2.8 pg)/tube, respectively, the intraassay coefficients of variation ranged from 3.54 to 9.30%, the interassay C.V. varied from 6.72 to 19.7%, depending on the type of method and on genistein concentration. The cross-reactivities with other chemically related compounds (with exception of genistein derivatives at the position used for construction of the immunogen) were 5.5 and 6.1% for daidzein and 3.9 and 0.04% for formononetin in RIAs using reagents prepared through positions 4′- and 7- of genistein, respectively. The method was used for measurement of genistein levels in 26 omnivore subjects and in three volunteers after consumption of a meal prepared from 125 g of cooked whole soybeans. The values obtained in ether extracts from human sera were almost identical for both RIA systems, indicating that both RIAs measure the same entity. © **1998 Elsevier Science Ltd. All rights reserved.**

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INTRODUCTION

Isoflavonoids are abundant in different leguminous plants, protecting them from pathogens and mediating their chemical communication with symbiotic Rhizobacteria [1]. Some isoflavonoids play important physiological roles in plant-consuming macroorganisms.

Phytoestrogens attracted the attention of veterinary physicians as early as 50 years ago, due to their adverse effects on sheep and cattle fertility when consuming huge amounts of phytoestrogen-rich pasture [2]. During the last decade an interest in the study of these compounds has increased considerably due to their potential effects as health-protecting dietary factors (for review see Ref. [3]).

Genistein (5,7,4′-trihydroxyisoflavone) belongs to the group of isoflavone-derived phytoestrogens. Besides its weak estrogenic activity, genistein is a well known inhibitor of tyrosine protein kinases, often used as a model compound in enzymologic studies [4]. The major source of genistein in the human diet is soya, although numerous less rich sources of this isoflavone are found [5, 6].

Genistein has previously been determined in human biological fluids by gas chromatography-mass spectrometry (GC-MS) or by high performance liquid chromatography (HPLC) [7–9]. Here we compare two RIA systems for the estimation of free genistein in human serum. The first system is based on polyclonal antisera against a conjugate of bovine serum albumin with genistein 4′-(carboxymethyl)ether (G4′-), the second one uses polyclonal antibodies against genistein 7-(carboxymethyl)ether conjugate with the same carrier protein (G7-). Bridge and position homologous conjugates of genistein with iodinated tyrosine methylester were used as radioligands. The G4′-RIA has already been used for measurement of genistein and biochanin A in beer [23], the G7-RIA is described for the first time.

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MATERIALS AND METHODS

Chemicals

4′-*O*-Carboxymethylgenistein was prepared by selective alkylation of the phenolic 4′-hydroxy group of genistein using potassium-tert-butoxide and ethyl bromoacetate in dimethylformamide. After alkylation the ethyl ester was hydrolyzed by treatment with base giving the free acid in good yield.

The synthesis of 7-*O*-carboxymethylgenistein was as follows: dry genistein was reacted with anhydrous potassium-tert-butoxide and bromoacetic acid ester at 100°C in dry dimethylformamide under argon atmosphere for 5 h. The reaction gave 7-*O*-(ethoxycarbonylmethyl) genistein in good yield. The ester was hydrolyzed in acidic conditions furnishing 7-*O*-(carboxymethyl)-4′,5-dihydroxyisoflavone (i.e. 7-*O*-carboxymethylgenistein) in a quantitative yield [10].

Daidzin was a generous gift of Dr Takaaki Yasuda from Tohoku College of Pharmacy, Miyagi, Japan. Equol has been prepared in the Institute of Organic Chemistry, University of Helsinki [11].

Bovine serum albumin (BSA) was from Bioveta (Ivanovice, Czech Republic) and casein from Difco Labs (Detroit, MI). Diethyl ether (stabilized with 0.001% fenidone) was from Synthesia (Pardubice, Czech Republic). All other organic solvents were from Merck (Darmstadt, Germany). Dicyclohexylcarbodiimide (DCC) and chloramine-T were from Fluka (Buchs, Switzerland), *N*-hydroxysuccinimide (NHS) and charcoal (Norit A) were from Serva (Heidelberg, Germany), tyrosine methyl ester (TME), complete Freund's adjuvant, dioctyl sulphosuccinate and standards used for the testing of cross-reactivity of antisera, genistin and flavonoids luteolin (3′,4′,5,7-tetrahydroxyflavone), quercetin (3,3′,4′,5,7-pentahydroxy-flavone and apigenin (4′,5,7-trihydroxyflavone) were from Sigma (St. Louis, MO) and dextran T-70 from Pharmacia (Uppsala, Sweden). All other substances were of analytical grade, from Lachema (Brno, Czech Republic).

Immunogen synthesis and immunization

The immunogens were synthesized according to Yatsimirskaya with minor modifications [12]. In brief: 10 mg of the respective genistein-(carboxymethyl)ether was left to react overnight with DCC and NHS (molar ratio 3:4:5) in 200 μl of anhydrous dimethylformamide (G4′-) or in the same volume of the mixture dimethylformamide:dimethylsulfoxide 1:1 (G7-). The following day, the reaction mixture was centrifuged to remove crystals of dicyclohexylurea and the supernatant was used for conjugation with BSA in a reversed micellar system. The starting molar ratio genistein:BSA was 80:1. BSA (20 mg) was dissolved in 1.5 ml 0.02 M bicarbonate buffer, pH 8.5. This solution was added dropwise to 10 ml of 0.3 M dioctyl sulphosuccinate in octane under continuous stirring. After the mixture became clear, the dimethylformamide solution of the active intermediate formed from the respective genistein-(carboxymethyl)ether was added. The mixture was stirred for an additional 24 h at ambient temperature. Genistein–BSA conjugates were isolated from the mixtures by precipitation with 3 vol. of cold acetone (−20°C) followed by centrifugation. The supernatant was removed and the sediment was dissolved in 2 ml of distilled water, filtered through a 0.22 μm Millipore filter and lyophilized. The hapten/carrier protein ratio of the conjugate was estimated by UV spectrometry at 258 nm [13] and by titration of free NH_2 groups by trinitrobenzenesulphonic acid [14], yielding 22.1 and 9.8 molecules of the hapten per one molecule of BSA for G4′–BSA and G7–BSA, respectively.

Rabbits were immunized and antisera collected using a standard procedure [15].

Synthesis of ^{125}I-labelled radioindicators

Genistein-(carboxymethyl)ethers were activated in the same way as described for the immunogen synthesis. The resulting crude preparation was diluted with dimethylformamide to a final concentration of 1.0 mg of genistein/ml. Tyrosine methyl ester was radioiodinated using the conventional chloramine T method [16]. Iodinated TME was extracted from the water phase with ethyl acetate, evaporated under a stream of nitrogen and then dissolved in anhydrous dimethylformamide. Activated genistein solution, 15 μl, was mixed with 5 μl of crude ^{125}I-TME (mixture of ^{125}I-TME and TME obtained after the iodination of 1 μg TME with approximately 10 MBq of $Na^{125}I$) in a conical borosilicate tube. The mixture was allowed to react overnight at ambient temperature. The reaction mixture was then chromatographed on a TLC-silica sheet (Merck, Art. 5583) in the system dichloromethane:2-propanol:acetic acid (96:4:0.5 v/v). The distribution of radioactivity on the chromatogram was scanned on a LB 285 Linear Analyzer (Berthold, Wildbad, Germany). The main peak was cut off and eluted with 3 ml of ethanol. The radioligand was stored in ethanol at −20°C.

An alternative synthetic approach was tested, based on the radioiodination of previously prepared conjugates of genistein with tyrosine methyl ester [17]. This approach was successful only for the G7–TME conjugate. Radioiodination of genistein TME derivatized at the 4′-position did not provide a useful radioligand.

Moreover, we also tried to prepare genistein radioiodinated directly to the isoflavone skeleton. The radiochromatogram of the mixture of products showed two peaks of radioactive material, which both moved faster than genistein in the straight phase chromatography, but none of them was useful as a ligand for any of the anti-genistein antibodies.

Specificity of the assay

Cross-reactivity of selected substances was expressed as a ratio of the 50% intercepts of the analyte and the respective cross-reactant.

HPLC of ether extract from sera after soybean consumption

The HPLC system consisted of an LC-6A pump (Shimadzu, Japan), a column oven LCO100, a UV detector LCD 2082 (Ecom, Czech Republic) and a fraction collector FC-203B (Gilson, France). An ET 250/4 Nucleosil 100-5 C18 (Macheray-Nagel, Germany) column was used. The mobile phase was methanol:water (60:40 v/v), the flow rate was 0.8 ml/min and the temperature was 35°C. The extract from 1 ml of sera obtained 4 h after soybean intake was chromatographed as described and 0.4 ml fractions were collected, evaporated at a speedvac evaporator and dissolved in 1.0 ml of the assay buffer. The 100 μl aliquots of individual HPLC fractions were assayed for immunoreactivity using G4′- and G7-RIA systems.

Sample extraction

Samples of 250 μl of sera were diluted with 250 μl of 0.9% saline and extracted with 1 ml of ether. The water phase was frozen in solid carbon dioxide–ethanol mixture and the ether phase was transferred into a glass tube. After thawing, the water phase was re-extracted and the ether extracts were combined. The solvent was evaporated and the dry residue was dissolved in 1 ml of assay buffer (20 mM sodium phosphate in saline, containing sodium azide and casein, 1.0 g/l each), so that 200 μl of the solution corresponded to 50 μl of the sample. Extracts of samples giving the signal outside the range of the calibration curve (5.88–1480 fmol, i.e. 1.59–400 pg/tube) were diluted with the assay buffer (therefore, the extraction was always performed from the same volume and dilution of sera). The standard curve prepared in the assay buffer was processed in the same way as were the samples.

The assay

The radioligand (10 000–15 000 cpm) and the antibody (working dilution 1:40 000 for G4′-RIA and 1:20 000 for G7-RIA, by volume) dissolved in the assay buffer were added to the tubes containing diluted samples and standards and the final volume was adjusted to 400 μl with buffer. After vortex-mixing and incubation overnight at 4°C, bound and free portions were separated by dextran–charcoal adsorption (0.5 ml of the suspension of 0.25 g charcoal and 0.025 g dextran T-70 in 100 ml assay buffer) and after additional incubation at 4°C for 15 min the tubes were centrifuged at 4°C for 10 min. Radioactivity in the supernatants was measured in a Berthold Twelve-Channel Gamma Counter (Wilbad, Germany). Each sample was assayed in duplicate.

Subjects

Morning serum samples were obtained from 26 Czech volunteers of both sexes. All the subjects were omnivores considering themselves to eat usual Czech meals. None of them ate soybeans at least the day before sample collection. Three volunteers were given a meal prepared from 125 g of soybeans (dry wt). Serum samples were collected before and 2, 4, 6, 8, 24 and 48 h after consumption of soybeans.

RESULTS

Specificity of the antisera

Cross-reactivities of selected isoflavonoids and flavonoids are shown in Table 1.

HPLC of ether extract from sera after soybean consumption

The chromatography of the ether extract from sera after soybean consumption showed one major immunoreactive peak corresponding to the position of genistein standard and a small peak at the position of the daidzein standard. Additionally, only the G7-RIA displayed one small peak with a higher chromatographic mobility than daidzein on C18-RP HPLC (more polar) and another small peak moving between formononetin and biochanin A (Fig. 1).

Reliability of the assay and parameters of the calibration

The sensitivity, expressed as a minimal amount of genistein distinguishable from the zero sample with 95% probability, was 4.44 fmol (1.2 pg) and 10.4 fmol (2.8 pg), the 50% intercept of the calibration curve 122 fmol (32.9 pg) and 251 fmol (67.8 pg)/tube, for G4′- and G7-RIAs, respectively.

Table 1. Cross-reactivity of the sera against geinistein-4′- and genistein-7-

Compound	Antiserum No. 14, crossreaction (%)	Antiserum No. 23, crossreaction (%)
Genistein	100.00	100.00
Genistin	0.00	26.4
Biochanin-A	172.90	0.73
Daidzein	5.50	6.10
Daidzin	0.00	1.24
Dihydrodaidzein	0.36	0.36
Formononetin	3.90	0.036
Equol	0.00	0.00
Apigenin	0.00	0.00
Luteolin	0.00	0.00
Quercetin	0.00	0.00
O-desmethylangolensin	0.00	0.00

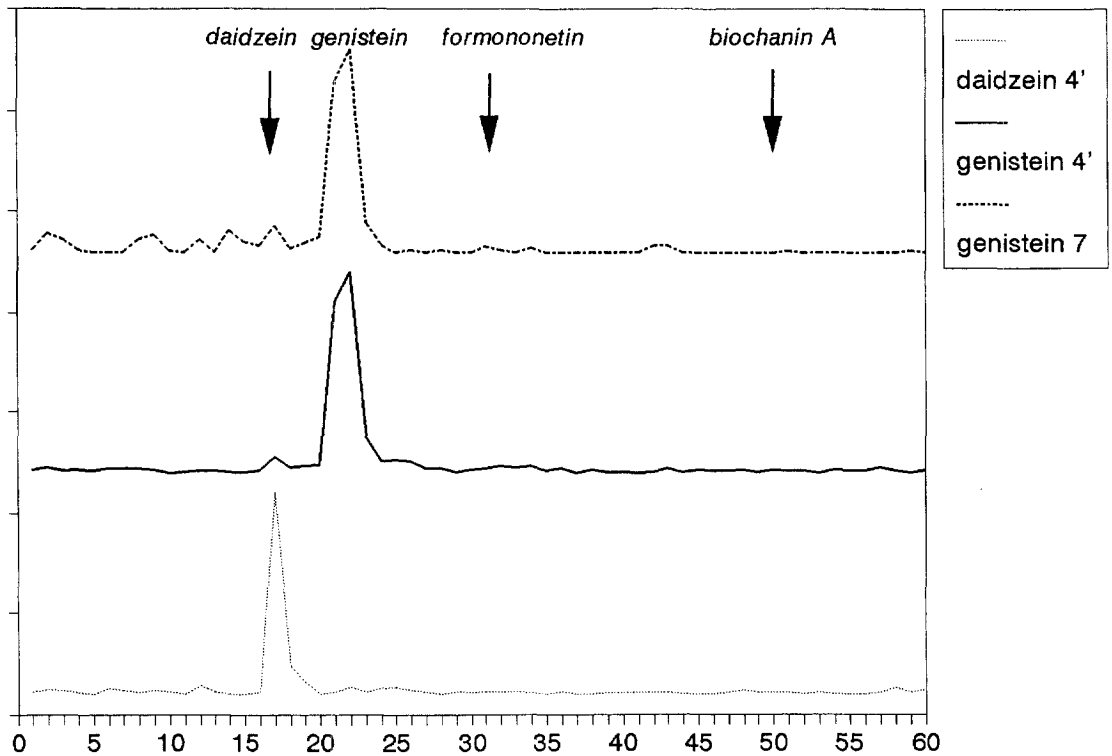

Fig. 1. Immunoreactivity of individual HPLC fractions of ether extract from human serum after soybean consumption. The arrows indicate the position of individual isoflavonoids on the chromatogram. In the lower part of the figure, the immunoreactivity of daidzein using an analogous RIA system for daidzein-4′ derivatives is shown (Lapčík, 1997).

The working range of the assay was 5.77–1480 fmol (1.56–400 pg)/tube.

Five sera were analyzed in days for determination of the intra- and interassay coefficients of variation. The intraassay coefficients of variation, determined from quadruplicate parallel analyses varied from 5.1 to 9.8% (mean 7.1%) and from 3.54 to 8.53%, (mean 7.3%) for the G4′- or G7-RIA system, respectively (Table 2). For the interassay coefficients of variation, the corresponding values 5.4–14.8% (mean 8.8%) and 6.7–19.7 (mean 10.3%), respectively, were obtained (Table 3).

The results did not depend on the dilution of serum extracts within the range of the calibration curve. The recovery of standard added to the serum was only 66.6–84.1% (mean, 73.4%; SD, 5.7%) regardless whether the G4′- or G7-RIA system was used (Table 4).

Free serum genistein levels in control subjects and after the consumption of soybeans

Genistein levels in 26 Czech omnivore volunteers varied from zero to 1.24 nmol/l when assayed with the G4′-RIA (mean, 0.22 nmol/l; SD, 0.30 nmol/l; median, 0.10 nmol/l) and from zero to 1.67 nmol/l when assayed with G7-RIA (mean, 0.21 nmol/l; SD, 0.36 nmol/l; median, 0.08 nmol/l). As shown on Fig. 2, after consumption of 125 g of cooked soybeans the serum levels of genistein reached the peak 4 h later. As demonstrated in Fig. 3, both methods closely correlated.

DISCUSSION

Genistein is a natural compound abundant in certain foods. It was thought to be one of the dietary health-protecting factors responsible for a lower inci-

Table 2. Intraassay coefficients of variation

Sample	G4′-RIA (nmol/l)	C.V. (%)	G7-RIA (nmol/l)	C.V. (%)
R3	22.36	7.1	19.30	3.5
R4	27.04	5.1	27.71	8.5
M2	13.45	7.1	13.57	11.2
M4	39.80	6.5	42.37	5.2
Kuč	0.091	9.8	0.098	8.3

Table 3. Interassay coefficients of variation

Sample	G4'-RIA (nmol/l)	C.V. (%)	G7-RIA (nmol/l)	C.V. (%)
O2	24.33	6.7	23.76	7.2
O4	22.60	14.8	29.39	6.7
M2	15.52	9.2	14.37	8.4
M4	39.80	5.4	40.41	9.4
Kuč	0.098	8.0	0.16	19.7

Table 4. Recovery of genistein added to a charcoal stripped serum

Genistein added (pg)	G4'-RIA genistein recovered (pg)	Recovery (%)	G7-RIA genistein recovered (pg)	Recovery (%)
6.25	4.43	70.9	4.34	69.4
12.5	9.56	76.3	9.13	73.6
25.0	17.04	68.2	20.96	83.8
50.0	33.32	66.6	35.70	71.4
100.0	74.25	74.3	84.05	84.1
200.0	136.99	68.5	146.90	73.5

dence of certain cancers and cardiovascular diseases in vegetarians and in Asians when compared with Europeans consuming a usual 'western' diet. The beneficial effect of this isoflavonoid seems to be based on its generally anti-mitotic and anti-proliferative effects rather than on its weak estrogenic and/or anti-estrogenic properties (see Refs. [18, 19]).

The analytical methods for studying the bioavailability and metabolic fate of dietary flavonoids and isoflavonoids are based on the combination of gas chromatography (GC) or high performance liquid chromatography (HPLC) with mass spectrometry [7, 8]. Recently, Franke *et al.* described a HPLC-diode array system for estimation of total isoflavonoids in body fluids [9]. These methods usually require time-consuming pre-processing of samples and special equipment, rendering them laborious and expensive. An immunoassay could be a powerful alternative to these methods, thanks to its ease, relatively low price and its availability for serial work.

Immunoassays based on antisera against derivatives differing in the position used for the construction of the immunogen in combination with suitable extraction methods could easily discriminate between free isoflavonoids and their different metabolites. The immunoassay based on the G7-antisera may be useful for the screening of genistein in food and plant material, because the predominant form of genistein in these sources is its 7-O-glucoside, genistin [1, 20]. Although non-isotopic methods should be preferred for routine work, it is not unusual that isotopic

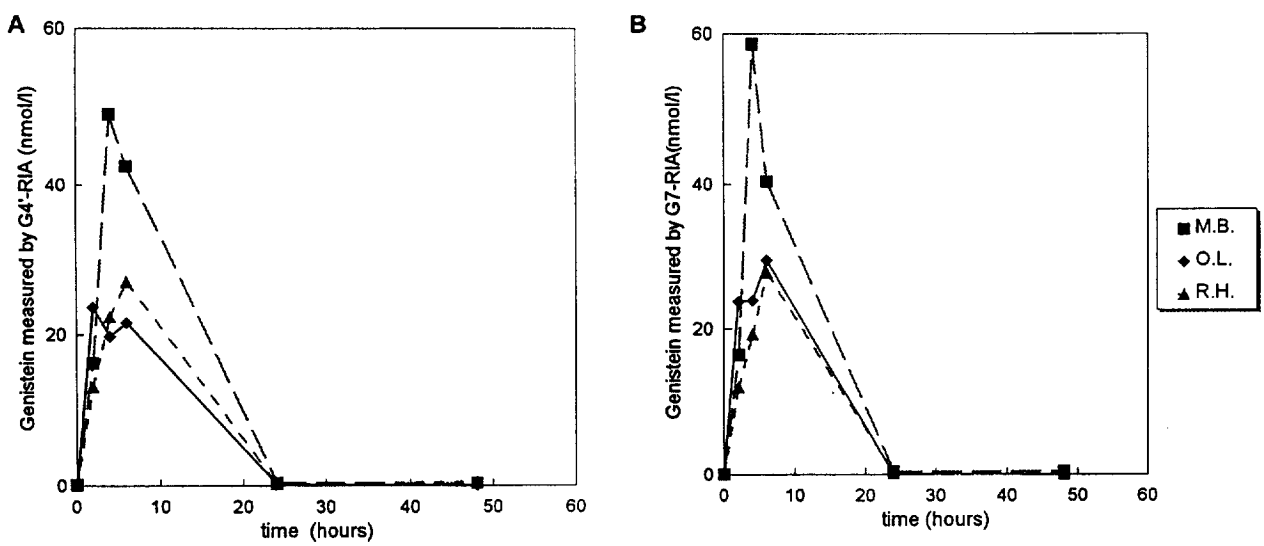

Fig. 2. Serum levels of free genistein in three volunteers after consumption of 125 g of cooked whole soybeans. (A) The levels found using the G4'-RIA and (B) the levels found using the G7-RIA.

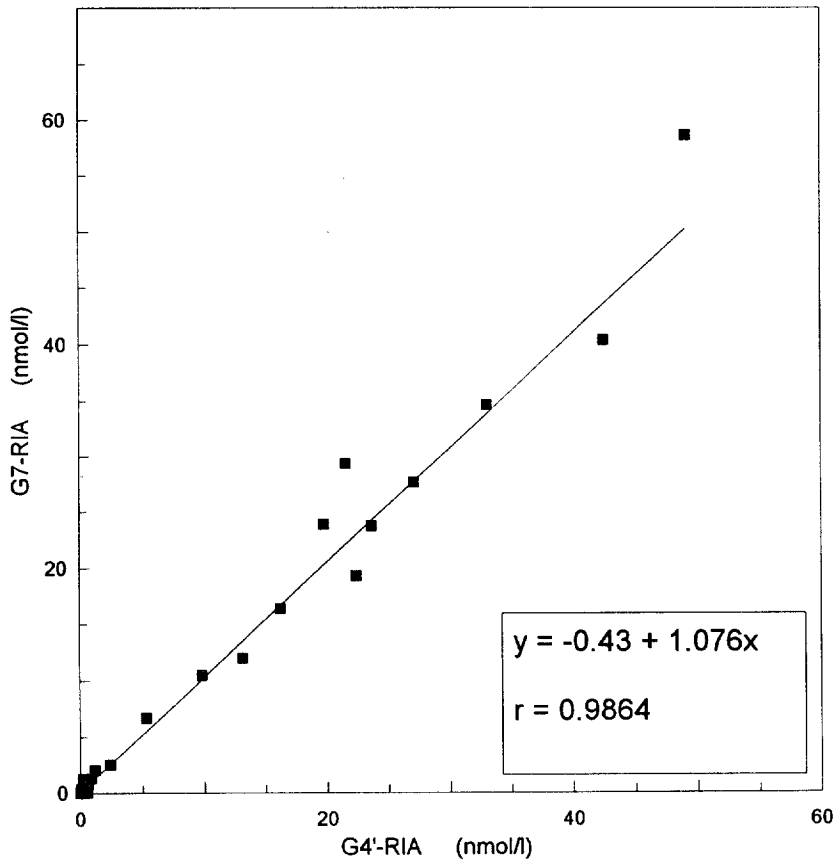

Fig. 3. Correlation between results obtained from analyzing the ether extracts from sera of 26 omnivores and three subjects after consumption of cooked whole soybeans, using the G4′- and G7-RIA systems.

methods are developed earlier. Non-radioactive variants of the same immunoassays have already been started to be developed.

As we expected, the G4′-RIA did not distinguish between genistein and its 4′-methoxy derivative, biochanin-A, methoxylated at the position through which the hapten was attached to the carrier protein in the immunogen. Analogously, the G7-RIA system does not distinguish between genistein and genistin. It may be expected that other 4′-*O* derivatives of genistein would elicit a signal of the same order of magnitude in the G4′-RIA, and analogously, 7-*O* derivatives of genistein would be recognized by the G7-RIA. Appropriate extraction methods thus might allow us to measure either free genistein only or the sum of free genistein and its monoglucuronides and monosulphates conjugated at the position used for construction of the immunogen. Sulphates and glucuronides are not extracted into ether.

The radioimmunossay of HPLC fractions of ether extracts from sera after soybean consumption showed one major immunoreactive peak corresponding to the position of genistein.

The cross-reactivities of the antisera used in the methods with daidzein, formononetin and biochanin A are not negligible and should be taken into account in individual applications. It does not seem to be a problem in the case of the genistein estimation in body fluids of subjects consuming soy-products, because both isoflavonoids are present in similar concentrations in soya [5, 20–22]. The results of the genistein estimation may be slightly overestimated in such materials, but a positive bias of several percent is usually still acceptable. On the other hand, some special applications could require a separation step before immunoassay. Recently we have measured daidzein, genistein, formononetin and biochanin-A in beer by immunoassay after HPLC separation of individual isoflavonoids [23]. However, even in this case, the whole procedure is easier and more sensitive than the corresponding GC-MS methods [24].

The close correlation between G4′- and G7-RIAs indicates that both methods measure the same entity in the ether extracts from sera. Since the methods differ in their cross-reactivities, only genistein can be the measured entity. The concentration of biochanin A, which is recognized only by the G4′-RIA, is low in soybeans [5, 20] and it is converted to genistein in the intestines. When ethyl acetate was used for the extraction instead of ether, the G7-RIA overestimated the genistein levels in comparison with the G4′-RIA in some samples, thus indicating that some more

polar metabolite of genistein could be partially extracted to ethyl acetate (data not shown).

The genistein concentrations in sera of control subjects were very low, usually close to or even below the detection limit. This is not very surprising, because the usual diet in the Czech Republic is not rich in these compounds. It has to be noticed that the free genistein serum levels after soybean ingestion were only tens of nmol/l, whilst previous GC studies describe levels of a micromolar order of magnitude for total genistein in such situations [25]. This is caused by a rapid conversion of genistein to its more polar metabolites in the human organism. We have recently described RIA for daidzein based on antibodies against the 4′-*O*-derivative of daidzein. The free daidzein in the same set of samples represented only 8.3% of the total immunoreactivity in directly assayed serum [26]. A strong matrix effect of serum with regard to genistein did not allow us to use the same approach for genistein and to measure total G4′- and G7-immunoreactivities in diluted serum. The attempt to find simple but reliable conditions for the immunoassay of polar metabolites of genistein continues.

Flavonoids and isoflavonoids are supposed to be excluded from animal organisms rather fast without forming any residues [27]. There are two principal ways of metabolising these compounds before their excretion, namely conjugation either with glucuronic acid or with sulphuric acid. Whilst sulphates may still be converted back to free isoflavonoids by the action of localized tissue sulphatases, glucuronides are considered to be inactive excretion forms of isoflavonoids. The immunoassays sensitive to position-specific derivatives of isoflavonoids could be in future useful tools for detailed study of their metabolic fate. A better knowledge of the turnover of these dietary factors could be important with respect to a potential use of genistein and/or related compounds in prevention of the diseases where a beneficial effect of these compounds has been established.

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