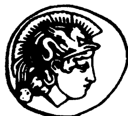

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Time-resolved fluoroimmunoassay for equol in plasma and urine

Elke Brouwers [a], Rafaëlla L'homme [a], Nawaf Al-Maharik [b,1], Oldrich Lapcík [c], Richard Hampl [d], Kristiina Wähälä [b], Heikki Mikola [e], Herman Adlercreutz [a,*]

[a] *Folkhälsan Research Center, Division of Clinical Chemistry, Institute for Preventive Medicine, Nutrition and Cancer, P.O. Box 63, University of Helsinki, FIN-00014 Helsinki, Finland*

[b] *Laboratory of Organic Chemistry, Department of Chemistry, P.O. Box 55, University of Helsinki, FIN-00014 Helsinki, Finland*

[c] *Department of Chemistry of Natural Compounds, Institute of Chemical Technology, Technicka 5, 166 28 Prague 6, Czech Republic*

[d] *Institute of Endocrinology, 116 94 Prague 1, Czech Republic*

[e] *Wallac Oy, P.O. Box 10, FIN-20101 Turku, Finland*

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Abstract

We present a method for the determination of the isoflavan equol in plasma and urine. This estrogenic isoflavan, which is formed by the action of the intestinal microflora, may have higher biological activity than its precursor daidzein. High urinary excretion of equol has been suggested to be associated with a reduction in breast cancer risk. The method is based on time-resolved fluoroimmunoassay, using a europium chelate as a label. After synthesis of 4′-*O*-carboxymethylequol the compound is coupled to bovine serum albumin (BSA), then used as antigen to immunize rabbits. The tracer with the europium chelate is synthesized using the same 4′-*O*-derivative of equol. After enzymatic hydrolysis (urine) or enzymatic hydrolysis and ether extraction (plasma) the immunoassay is carried out. The antiserum cross-reacted to variable extent with some isoflavonoids. For the plasma method the cross-reactivity does not seem to influence the results, which were highly specific. The overestimation of the values using the urine method (164%) compared to the results obtained by a gas chromatography–mass spectrometry (GC–MS) method is probably due to some influence of the matrix on the signal, and interference of structurally related compounds. It is suggested that plasma assays are used but if urine samples are measured a formula has to be used to correct the values making them comparable to the GC–MS results. The correlation coefficients between the time-resolved fluoroimmunoassay (TR-FIA) methods and GC–MS methods were high; *r*-values for the plasma and urine method, were 0.98 and 0.91, respectively. The intra-assay coefficient of variation (CV%) for the TR-FIA plasma and urine results at three different concentrations vary between 5.5–6.5 and 3.4–6.9, respectively. The inter-assay CV% varies between 5.4–9.7 and 7.4–7.7, respectively. The working ranges of the plasma and urine assay are 1.27–512 and 1.9–512 nmol/l, respectively.
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Keywords: Immunoassay; Phytoestrogens; Isoflavones; Urine; Equol; Fluorometry

1. Introduction

Isoflavonoids are a group of diphenolic (or better bisphenolic) hormone-like compounds of dietary origin that are of great interest particularly because of their anti-carcinogenic potency, but also because of their association with other Western diseases like coronary heart disease [1–3]. The isoflavonoids occur mainly in soybean products and clover seeds and leaves [4–6].

The isoflavonoids, one group of the phytoestrogens, have received increasing attention because of their many biological activities like radical scavenging, antioxidant [7], direct and indirect antiestrogenic [8–15], cytostatic, antiproliferative, differentiation inducing, and angiogenesis-inhibiting properties [16–21].

Abbreviations: GC, gas chromatography; HPLC, high performance liquid chromatography; MS, mass spectrometry; RIA, radioimmunoassay; TR-FIA, time-resolved fluoroimmunoassay; DELFIA, dissociation-enhanced lanthanide fluoroimmunoassay; EIA, enzyme immunoassay; FIA, fluoroimmunoassay; BSA, bovine serum albumin; TLC, thin-layer chromatography; DCC, *N,N′*-dicyclohexylcarbodiimide; NHS, *N*-hydroxysuccinimide; ID-GC–MS-SIM, isotope dilution gas chromatography–mass spectrometry in the selected ion monitoring mode; CVs, coefficients of variation; *B*, binding of tracer to antibody in the presence of unlabeled analyte; B_o, binding of tracer to antibody in the absence of unlabeled analyte; *T*, total amount of tracer added

* Corresponding author. Tel.: +358-9-1912-5380; fax: +358-9-1912-5382.

E-mail address: herman.adlercreutz@helsinki.fi (H. Adlercreutz).

[1] Present address: School of Chemistry, The Purdie Building, The University of St. Andrews, St. Andrews, Fife KY16 9ST, Scotland, UK.

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When consumed, isoflavonoids are converted by the intestinal microflora to biologically active secondary plant metabolites. The gut bacteria rapidly hydrolyze ingested glycosides, whereafter the aglycones undergo further metabolism. In this way the aglycone daidzein, which is formed from formononetin, is reduced by the intestinal microflora to the isoflavan equol (about 70%) and *O*-desmethylangolensin (about 5–20%) [22–26]. In sheep the aglycone genistein, which is formed from biochanin A, is metabolized to *p*-ethylphenol [27,28]. Studies have shown that the considerable inter-individual variation in the occurrence of isoflavonoids in human biological fluids is probably due to differences in metabolism, caused by different internal and external conditions [29–32]. For example, only 20–40% of the population is able to produce equol in larger amounts from daidzein after soy intake [29,31–33], but human urine may contain small amounts of equol derived from ingested milk and meat [34].

Equol, which was shown to be the estrogenic agent responsible for clover disease in sheep [35], was identified in human urine for the first time in 1982 [36,37]. Recently it was shown that equol has higher anti-carcinogenic potency, both in vitro and in vivo, than daidzein itself. A recent in vitro study with MCF-7 estrogen-dependent breast cancer cells showed that equol was more potent than daidzein in competing with estradiol for binding to the estrogen receptor. Equol also appeared to be 100 times more effective in causing an estrogenic response. Moreover, it was observed that equol, and not daidzein, reduced the mRNA expression of the estrogen-responsive *pS2* gene in the MCF-7 cells [38]. An earlier assay based on the estrogen-specific enhancement of the activity of alkaline phosphatase in human endometrial tumor cells, showed a higher estrogenic activity of equol compared to daidzein [39]. Equol has higher antioxidant activities, being a more potent inhibitor of lipid peroxidation in liposomes induced by Fe(II) or Fe(III). This could be due to the absence of the 2,3-double bond, and the 4-oxo group in equol [7,40]. In addition to these in vitro results, a recent epidemiological study, carried out in breast cancer patients, showed that high urinary excretion of equol was associated with a significant reduction in breast cancer risk, but no association with daidzein excretion was found [41]. A similar tendency was observed already in 1982 [37]. The biological activity of equol and the association of the excretion of this compound with a decrease in breast cancer risk [42] make the compound very attractive for further studies.

Available methods for measuring isoflavones and their metabolites in human plasma and urine have in the past been based on gas chromatography–mass spectrometry (GC–MS) [25,43–48] and high performance liquid chromatography (HPLC) [49–51]. Especially the GC–MS method is very expensive and time consuming. Although the HPLC method is more convenient and less expensive, it has, in general, a lower specificity and sensitivity than GC–MS. When dealing with large screening studies, these methods are not well suitable.

Antibodies against some phytoestrogens have been raised as early as in 1969 in rabbits to be used in sheep for passive immunization as protection against an excess of phytoestrogens, which have a major role in reproductive dysfunction in sheep [52]. A radioimmunoassay (RIA) for formononetin in plasma and rumen fluid of wethers fed on red clover was already presented [53]. Two radioimmunoassays for unconjugated and total daidzein and genistein in human biological fluids have earlier been developed [54,55]. The potential hazards, although slight, and the short shelf life of the radioactive labeled compounds led to the development of even more simple methods based on time resolved fluoroimmunoassay. This method combines the advantages of other nonradioisotopic assays (stability, lack of radiation, no waste problems) with a 10–100-fold increase in sensitivity and assay range compared to conventional enzyme immunoassay (EIA) and fluoroimmunoassay (FIA) methods. Moreover time-resolved fluoroimmunoassay (TR-FIA) has low background interference.

The excellence of TR-FIA in routine immunodiagnostic work, as employed in the dissociation-enhanced lanthanide fluoroimmunoassay (DELFIA) system by Wallac Oy, is already well established. In the DELFIA system, europium is bound to the analyte using a nonfluorescent chelate. After completion of the bioaffinity reaction, the europium ions are dissociated from the chelates by means of an enhancement solution in which lanthanide ions form highly fluorescent complexes with components of this solution. The enhanced fluorescence is measured at a fixed time after excitation of the fluorophore. By this time, the background fluorescence has died away.

TR-FIAs for enterolactone, daidzein and genistein in human urine and plasma have already been developed [56–59]. In the present study we developed TR-FIA methods for equol in human plasma and urine. Results were confirmed using previously published GC–MS methods for plasma [47] and urine [44,60]. Conditions were optimized and methodological parameters, i.e. accuracy, precision, sensitivity and specificity, were determined.

2. Materials and methods

2.1. Instrumentation

TR-FIA for equol was performed with the following instruments and related reagents, all purchased from Wallac Oy (Turku, Finland): a Victor 1420 multilabel counter with software version 1.0 for fluorescence measurements and a LKB 1217 Rackbeta liquid scintillation counter for radioactivity counting, DELFIA enhancement solution to develop the fluorescence, and white goat anti-rabbit IgG coated microtitration strips, DELFIA plate wash, DELFIA plate shake, and DELFIA wash solution for immunoassay procedures.

HPLC purification of the europium-labeled equol derivative was performed using Gradient Pump 2249, UV-monitor

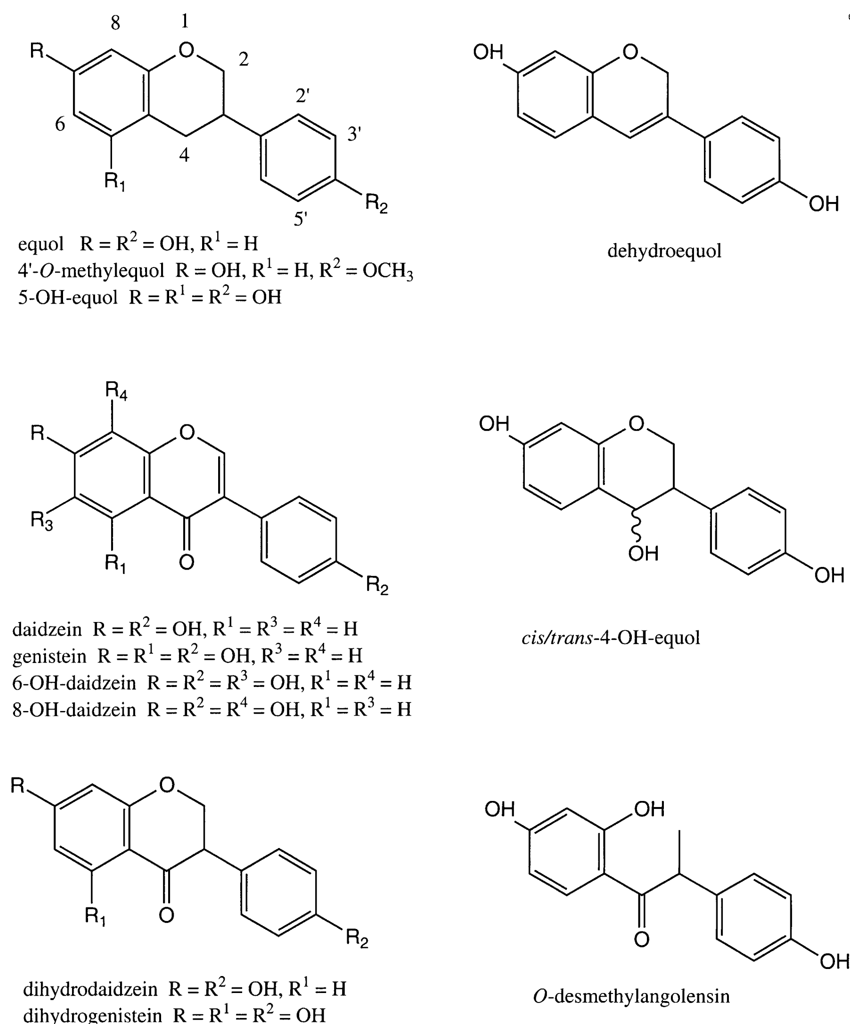

Fig. 1. Structures of isoflavonoids tested on cross-reactivity.

Uvicord SII, and Superdex Peptide column, all from Pharmacia (Uppsala, Sweden).

2.2. Chemicals

Standards: The 13 compounds studied in the cross-reactivity experiments were equol, daidzein, genistein, dihydrodaidzein, dihydrogenistein, *O*-desmethylangolensin, dehydroequol, 5-OH-equol, *trans*-4-OH-equol, *cis*-4-OH-equol, 4′-*O*-methylequol, 6-OH-daidzein and 8-OH-daidzein. The structures of these compounds are shown in Fig. 1. Equol was prepared from daidzein by hydrogenation in ethanol with palladized charcoal and hydrogen gas [61]. Daidzein was synthesized by a one-pot procedure starting from appropriately substituted resorcinol and phenyl acetic acid using boron trifluoride etherate as Lewis catalyst and solvent and dimethylformamide as a carbon source [62,63]. Genistein was purchased from Karl Roth GmbH (Karlsruhe, Germany). Dihydrodaidzein and dihydrogenistein were synthesized from the corresponding isoflavones daidzein and genistein by reducing selectively the carbon–carbon double bond in the C-ring by diisobutylaluminiumhydride [64]. *O*-Desmethylangolensin was synthesized from 2-(*p*-methoxyphenyl) propionic acid and 1,3-dimethoxybenzene in polyphosphoric acid followed by demethylation using 1.0 M borontribromide in dichloromethane [65]. Dehydroequol was a generous gift from Dr. Andy Liepa. 5-OH-equol was obtained by catalytic hydrogenation of genistein [66]. *trans*-4-OH-equol and *cis*-4-OH-equol were synthesized from dihydrodaidzein by lithiumborohydride in tetrahydrofuran at 0 °C under argon [67]. Formononetin was hydrogenated catalytically by Pd/C and H_2 gas in aqueous ethanol to give 4′-*O*-methylequol in good yield [68]. Glycitein was demethylated by borontribromide in dichloromethane to furnish 6-OH-daidzein [69]. 8-OH-daidzein was prepared by cyclisation of 3,4,4′-trihydroxybenzoin, which was synthesized from *ortho*-hydroxyphenol and 4′-hydroxyacetic acid by Friedel-Crafts acylation [62].

Reagents: Bovine serum albumin (BSA) and diethylether were of analytical grade (Merck AG, Darmstadt, Germany). Methanol was purchased from Rathburn Chemicals Ltd. (Walkerburn, Scotland, UK). β-Glucuronidase (EC 3.2.1.31) (Boehringer Mannheim, Mannheim, Germany; Cat. no. 1585665), DDC and *N*-hydroxysuccinimide (NHS) were from Sigma (St. Louis, USA) as well as sulfatase (EC 3.1.6.1) (Cat. no. S9626). [6,7-^{3}H]Estradiol-17β-17-glucuronide (specific activity 1.9 TBq/mmol (51 Ci/mmol)) was from NEN Lifescience Products (obtained from Wallac Oy).

2.3. Buffers

The assay buffer used consisted of 50 mmol/l Tris–HCl buffer, pH 7.8, containing 8.78 g of NaCl, 0.5 g of sodium azide, 5 g of BSA, and 0.1 g of Tween 40 per liter. The 0.1 mol/l acetate buffer, pH 5.0, was used in enzyme hydrolysis of equol conjugates. The same assay buffer was used for cross-reactivity experiments and for preparation of the equol standard solution, used for the standard curve.

2.4. Synthesis of 4′-O-carboxymethylequol

4′-*O*-Ethoxymethyldaidzein [70] was reduced by palladized charcoal and hydrogen gas in ethanol in a hydrogenation apparatus. The hydrolysis of the corresponding ethyl ester (4′-*O*-ethoxycarbonylmethyl-equol) with 10% KOH in aqueous methanol provided the desired 4′-*O*-carboxymethylequol in good yield. [71].

2.5. Immunogen synthesis and immunization

The immunogen was synthesized according to Yatsimirskaya et al. with minor modifications [72]. One and a half milligrams of 4′-*O*-carboxymethylequol was reacted overnight with 1.5 mg *N,N*′-dicyclohexylcarbodiimide (DCC) and 1.1 mg NHS in 80 μl of anhydrous dimethylformamide at an ambient temperature. The reaction mixture was centrifuged to remove the crystals of dicyclohexylurea, and the supernatant was used for conjugation with BSA. Five milligrams of BSA was dissolved in 375 μl of 0.01 mol/l sodium bicarbonate buffer pH 8.5. This solution was added drop wise to 3 ml of 0.3 mol/l dioctylsulfosuccinate in octane under continuous stirring. After the mixture became clear, the dimethylformamide solution of the active intermediate formed from 4′-*O*-carboxymethylequol was added. The mixture was stirred an additional 24 h at ambient temperature. The hapten–BSA conjugate was isolated from the mixture by precipitation with 3 volumes of cold acetone (−20 °C) followed by centrifugation. The supernatant was discarded, and the sediment was washed in 3 ml of cold acetone and centrifuged again. The sediment was dissolved in 1.0 ml of distilled water, filtered through 0.22 μm Millipore filter and lyophilized. Thereafter, rabbits were immunized and sera were collected using a standard procedure [73].

2.6. Labeling of the equol derivative with europium chelate

4′-*O*-Carboxymethylequol was labeled in 0.5 mol/l 4-morpholinoethanesulfonic acid buffer, pH 5.5, with 4-aminobenzyldiethylenetriaminetetraacetic acid europium chelate [74] using 1-(3-(dimethylamino) propyl) ethyl carbodiimide hydrochloride as condensation agent, and purified on a preparative thin-layer chromatography (TLC) plate, as previously described for steroid derivatives [75]. The labeled equol derivative was further purified by HPLC (eluent: 10% acetonitrile in 0.05 mol/l NaCl with 0.05 mol/l Tris–HCl).

2.7. Determination of equol by GC–MS

GC–MS methods with deuterated internal standard for plasma and urine were used to confirm TR-FIA results. For plasma the original method [47] was slightly modified. In the original method the free and sulfate fraction of the phytoestrogens (isoflavonoids and lignans) were separated from the glucuronides by ethyl ether extraction after separate hydrolysis of the sulfates. Thereafter, the glucuronides were hydrolyzed and then extracted by ethyl ether. Both fractions were then processed separately and analyzed finally using GC–MS. In the present study the hydrolysis and extraction were carried out, using the same conditions as for the TR-FIA samples. Samples were further purified for GC–MS analysis. The GC–MS method for urine is very complicated, because it also includes the determination of 14 estrogens and 7 other phytoestrogens. It has been described in detail previously [44,60].

2.8. Statistical treatment

The correlations between the results for the TR-FIA and GC–MS results were calculated using Excel 98 programs for Macintosh.

2.9. Pretreatment of plasma samples

Recovery was determined using [^{3}H]estradiol-17-glucuronide, because glucuronides of equol were not available. [^{3}H]Estradiol, corresponding to 30,000 cpm, was added to 200 μl of plasma. Samples were mixed and equilibrated for 30 min at room temperature. Two hundred microliters of 0.1 mol/l acetate buffer pH 5.0 containing 0.2 U/ml β-glucuronidase and 2 U/ml of sulfatase were added. Samples were mixed and incubated overnight at 37 °C. Hydrolyzed samples were extracted twice with 1.5 ml of diethyl ether using Vortex mixer. The ether phases were combined and evaporated to dryness (with nitrogen) in a 45 °C water bath. Samples were dissolved in 200 μl of assay buffer. After thorough mixing, 20 μl (in duplicate) was subjected to TR-FIA. The samples giving a value outside the range of the standard curve were further diluted with

the assay buffer. Another 20 μl of the sample was taken for liquid scintillation counting for determination of recovery.

For samples with very low equol concentration 450 μl plasma was pretreated using the same ratio of reagents. Samples were then dissolved in 150 μl of assay buffer.

2.10. Pretreatment of urine samples

Twenty microliters of urine was hydrolyzed with 180 μl of 0.1 m acetate buffer pH 5.0 containing 0.2 U/ml of β-glucuronidase and 2 U/ml of sulfatase incubating overnight at 37 °C. Then 300 μl of assay buffer was added and after mixing, 20 μl samples of the solution were analyzed by TR-FIA in duplicate.

2.11. Time-resolved fluoroimmunoassay

Twenty microliters of standard, hydrolyzed and extracted plasma, or hydrolyzed urine were pipetted into prewashed goat anti-rabbit IgG microtiter plate strips. To each well was added 100 μl of antiserum (dilution of 1:240,000 for plasma and 1:150,000 for urine) in 0.5% BSA Tris-buffer and 100 μl of europium-labeled equol diluted to a suitable concentration. After incubation and shaking the strips slowly on the DELFIA plate shaker at room temperature for 90 min, the strips were washed using the DELFIA plate washer. Then 200 μl enhancement solution was added to each well and the strips were shaken slowly for an additional 5 min. The enhanced fluorescence was measured in a Victor 1420 multilabel counter.

2.12. Calculation of the results

Calculation of the final result was done according to the formula: final result (nmol/l) = concentration (read in nmol/l) × 1/recovery (%) × dilution factor.

To correct for losses during the extraction the final results of plasma samples were calculated, using the recovery results (1/recovery (%)). The plasma samples were not diluted or concentrated (dilution factor = 1), unless they fell out of the standard curve. The urine samples were not extracted, so no correction had to be made. The final dilution of the urine samples was 1:25.

3. Results

3.1. Immunoassay optimization

In the competitive time-resolved fluoroimmunoassay for equol the europium-labeled and sample equol compete in binding to a limited amount of highly specific antibody. The assay was optimized for the amount of antibody and conjugate. The standard curve characteristics (detection limit, slope, and dynamic range) were optimal at antibody concentrations of 1:240,000, for equol in plasma, and at 1:150,000 for equol in urine. With these dilutions and in the absence of any unlabeled equol, the tracer occupied all the antibody-binding sites and 13.3 and 10.7% of the added tracer was bound (B_o) for plasma and urine, respectively. When unlabeled equol is present (standard or sample) it will compete with the tracer and the binding is determined (B). The nonspecific binding of the tracer reagent without the antibodies was <0.4 and <0.1% of the B_o values, for plasma and urine, respectively. We also investigated the effect of incubation time on the immunoassay. In 90 min, the maximum fluorescence signal generated reached a plateau. The standard solutions of equol in assay buffer made from a stock solution of equol in methanol are stable for 1 week when stored at 4 °C.

3.2. Specificity of the antiserum

Cross-reactivities of selected phytoestrogens are shown in Table 1. The percentage cross-reactivity for the equol antiserum was 0.0% for genistein and 10.0% for its main metabolite dihydrogenistein [23]. Daidzein cross-reacted 0.8% and its main metabolite dihydrodaidzein [23] 4.0%. The percentage cross-reactivity was 0.0% for 6-OH-daidzein and 8-OH-daidzein, 0.0% for *O*-desmethylangolensin, 10.7% for *cis*-4-OH-equol, 14.0% for 5-OH-equol, 42.3% for dehydroequol, 35.2% for *trans*-4-OH-equol and 275.0% for 4′-*O*-methylequol. The specificity studies using GC–MS are described in Section 3.3.

3.3. Accuracy

The recoveries of known amount of equol added to plasma samples ($n = 2$) (16, 128 and 512 nmol/l) were 95.8, 101.4 and 97.9%, respectively (mean 98.3%). The recoveries of known amount of exogenous equol added to urine samples ($n = 2$) (16 and 512 nmol/l) were 95.2 and 93.3%, respectively (mean 94.3%). The recovery was assessed by analyzing the samples with and without added equol, and then

Table 1
Specificity of equol antiserum

Compound	Equol antiserum percentage cross-reactivity
Equol	100.0
6-OH-daidzein	0.0
Genistein	0.0
8-OH-daidzein	0.0
O-Desmethylangolensin	0.0
Daidzein	0.8
5-OH-equol	14.0
Dihydrogenistein	12.0
Dihydrodaidzein	4.0
cis-4-OH-equol	10.7
Dehydroequol	42.3
trans-4-OH-equol	35.2
4′-*O*-Methylequol	275.0

Table 2
Intra- and inter-assay CVs for plasma equol assayed by TR-FIA in hydrolyzed and extracted plasma samples and hydrolyzed urine samples

Sample	Concentration (nmol/l)	Intra-assay CV%	Inter-assay CV%
Plasma method			
Low	4.5	6.5 ($n = 10$)	9.7 ($n = 10$)
Medium	52.6	5.5 ($n = 10$)	6.0 ($n = 10$)
High	133.7	6.3 ($n = 10$)	5.4 ($n = 10$)
Urine method			
Low	203.9	6.9 ($n = 10$)	7.7 ($n = 10$)
Medium	1663.5	3.4 ($n = 10$)	7.4 ($n = 10$)
High	6143.9	3.9 ($n = 10$)	7.6 ($n = 10$)

subtracting the concentrations of endogenous equol from the total value obtained after standard addition. Because in the plasma method the losses during hydrolysis and extraction are corrected for by the added tritiated estradiol glucuronide, we found it unnecessary to do more extensive studies. The accuracy can also be judged from the comparisons with the GC–MS method (see Section 3.4).

3.4. Precision

The results from the precision studies are shown in Table 2. Plasma samples with three different concentrations of equol (4.5, 52.6 and 133.7 nmol/l) were repeatedly analyzed 10 times either in one assay or in different assays. The intra-assay CV% was 6.5, 5.5 and 6.3% for the low, medium and high plasma sample, respectively. The inter-assay CV% were 9.7, 6.0 and 5.4%, respectively, for the low, medium and high plasma sample. The same was repeated with urine samples containing 204, 1663 and 6144 nmol/l of equol. The urine samples were measured after a 25 times dilution. The intra-assay CV% was 6.9, 3.4 and 3.9% for the low, medium and high urine sample, respectively. The inter-assay CV% was 7.7, 7.4 and 7.6%, respectively, for the low, medium and high urine sample. The mean and standard deviation of 10 standard curves obtained at different days, both for the plasma and urine method are shown in Figs. 2 and 3, respectively.

3.5. Sensitivity

The minimum amount of equol distinguishable from the zero samples with 95% probability was 6.2 pg/20 μl of plasma and 9.2 pg/20 μl of urine. The working range of the immunoassay for equol in plasma was from 1.3 to 512 nmol/l, corresponding to 6.2–2478 pg/20 μl of the plasma sample analyzed (Fig. 2). With the modification for plasma samples with very low concentration it is possible to measure plasma concentrations below 1 nmol/l. The working range of the assay for equol in urine after a 25 times dilution was from 1.9 to 512 nmol/l, corresponding to 9.2–2478 pg/20 μl of the urine sample analyzed (Fig. 3).

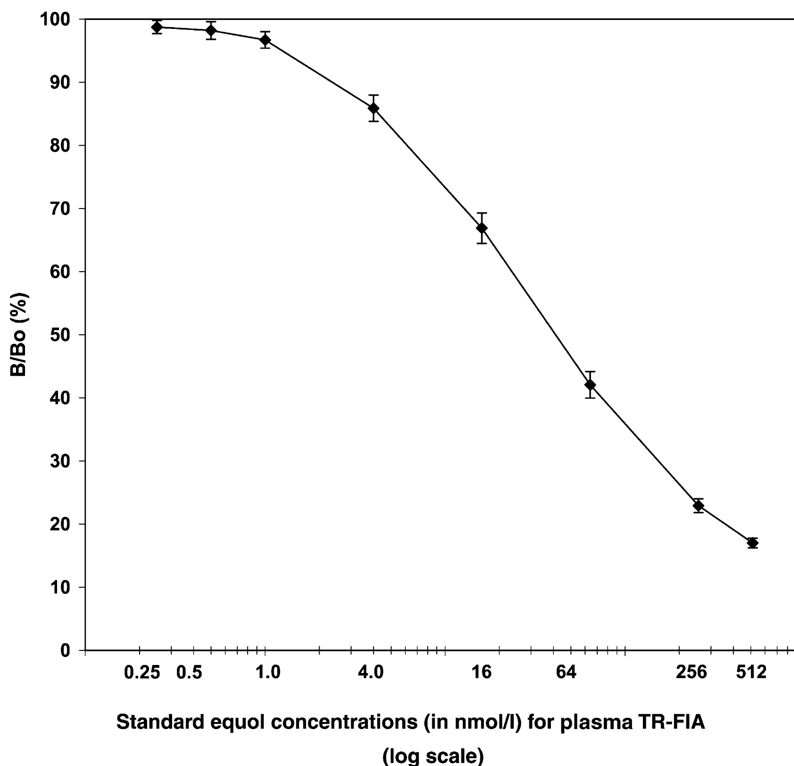

Fig. 2. Standard curve for equol plasma assay based on 10 separate standard curves on different days (±S.D.).

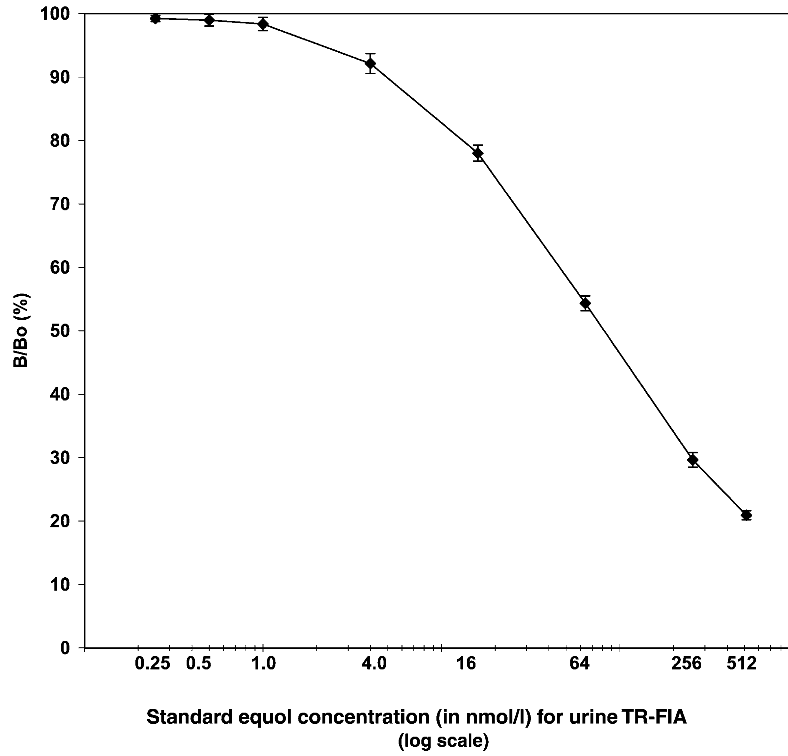

Fig. 3. Standard curve for equol urine assay based on 10 separate standard curves on different days (±S.D.).

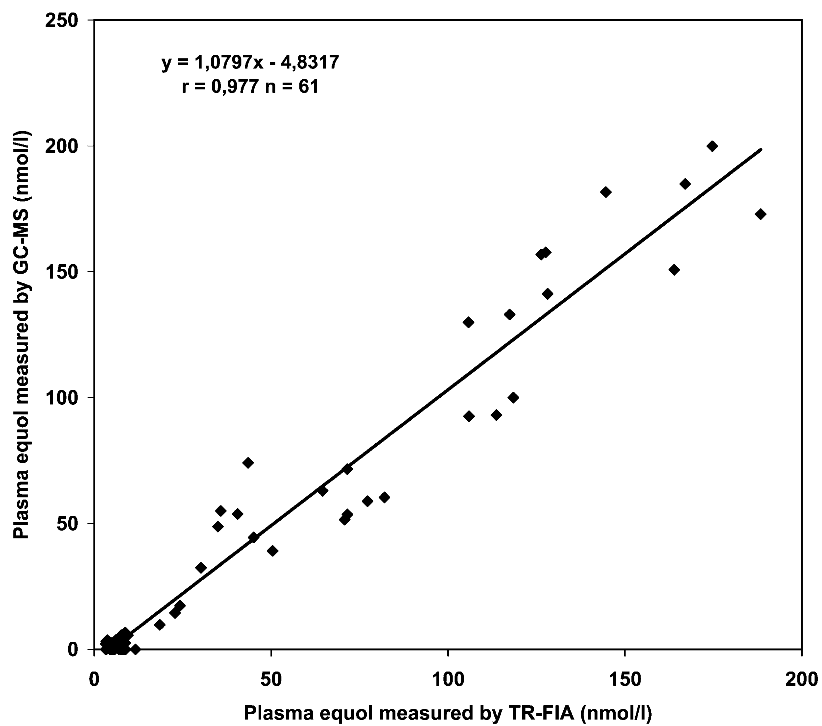

Fig. 4. Correlation between plasma equol measured by TR-FIA plasma method and measured by GC–MS plasma method.

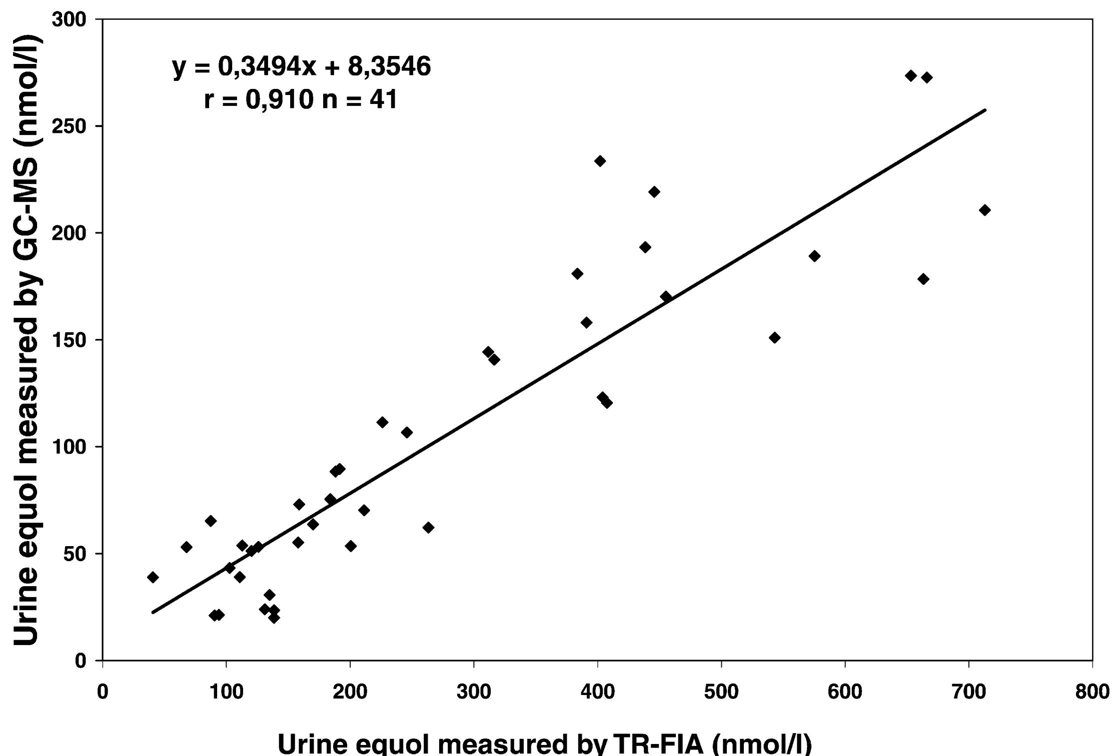

Fig. 5. Correlation between urine equol measured by TR-FIA urine method and measured by GC–MS urine method.

3.6. Methodological parameters of the GC–MS methods

The specificity, accuracy and sensitivity experiments for the plasma and urine GC–MS methods were described previously [44,47,60].

New precision values for equol were determined in connection with this study, because the previous values of precision were derived from samples with quantity of equol, which was too close to the detection limit. The intra-assay CV% for single determinations of plasma equol using this modification of the method was found to be 7.57% ($n = 10$; concentration 3.15 nmol/l) and 5.18% ($n = 10$; concentration 49.0 nmol/l). The first CV is measured in a sample with a concentration near to the detection limit, which influences the results. The inter-assay CV% for equol was 7.7% ($n = 11$) and 9.0% ($n = 12$), for samples with concentrations of 57.6 and 149 nmol/l, respectively.

The intra-assay CV% for single determinations of urine equol using the GC–MS method was found to be 7.01% ($n = 15$; concentration 19.4 nmol/l). The corresponding inter-assay CV% for equol was 9.18% ($n = 13$; concentration 20.7 nmol/l).

3.7. Correlation with GC–MS method

Correlations between the TR-FIA and the GC–MS method values were determined (Figs. 4 and 5). Samples from different sources, containing different amounts of equol were used and checked with both immunoassay and GC–MS. For TR-FIA, duplicate determinations of the final immunoassay of the samples were used and for GC–MS the values were based on single determinations.

The correlation coefficient between the TR-FIA plasma values and the GC–MS values was 0.977 (GC–MS result = 1.08 × TR-FIA result − 4.83) over the entire range of concentrations from 0 to about 200 nmol/l ($n = 61$). For the TR-FIA urine method the mean values of the duplicate determinations were approximately 164% higher than with the GC–MS method. The correlation between the two methods for urine is highly significant ($r = 0.910$; GC–MS result = 0.349 × TR-FIA result + 8.35) over the entire range of concentrations from 20 to about 300 nmol/l ($n = 41$). The TR-FIA values can be converted to GC–MS values using the above formula.

4. Discussion

There is an increasing interest in the biological role of the daidzein metabolite equol, particularly because of its possible association with breast cancer [37,41,42], and its high antioxidative potency [7]. Furthermore, the formation of equol in the human intestinal tract by the microflora is dependent on composition of the diet consumed by the subjects [33]. These interesting observations were the reason

for us to develop a rapid, convenient and specific method for this compound.

Most analytical methods to measure isoflavonoids and their metabolites are based on HPLC, GC or GC–MS and require time consuming pretreatment and high investments, which makes them too expensive and laborious for routine work and in large studies. Two groups have been working with the development of methods for the analysis of isoflavones including equol. Both methods are based on the ELISA technique and preliminary reports have been published. [76,77] The methodological details in these reports do not allow comparisons with the method described here and the sensitivity of the methods appear not sufficient for measurements in plasma at low levels. One of them [77] seems also to need a chromatographic step.

As expected, the antiserum against 4′-*O*-carboxymethylequol did not distinguish between equol and 4′-*O*-methylequol, which is a methyl ether derivative of equol, methylated at the (4′-*O*-) position through which the hapten was attached to the carrier protein in the immunogen. Probably this methyl group in 4′-*O*-position is easily lost in the gut as happens almost quantitatively with formononetin and biochanin A to result in daidzein and genistein, respectively. This demethylation results in formation of equol, which makes it less likely that 4′-*O*-methylequol would have any effect on the results. There is a very good correlation between the TR-FIA and GC–MS results for plasma (Fig. 4) suggesting that dihydrodaidzein and dihydrogenistein, despite their significant cross-reactivity with the antiserum, do not interfere much with assay specificity. However, for urine there probably is a significant cross-reactivity because of the abundance of these two metabolites, and the use of the suggested correction formula is indicated. This correction formula result in values comparable to those obtained with the reference GC–MS method. Anyway these cross-reactions should be taken into account when the method is used in applications where high dihydrogenistein and -daidzein levels are expected. *cis*-4-OH-equol and *trans*-4-OH-equol, which are only present in very low amounts in human urine samples [23], are probably of minor influence on the values obtained. The same is true for 5-OH-equol because this compound could not be found in urine [23]. It is unlikely that these compounds exist in plasma in measurable amounts. There are no published data on dehydroequol.

The precision values obtained in this study are satisfactory both for urine and plasma samples. A reason why the recovery of added standards for both the plasma and urine methods are lower than the theoretical value of 100% is probably due to the low concentration of equol in samples to which the standards were added approaching the sensitivity limit of the assay. At that level the precision is relatively poor and the values are easily slightly overestimated due to background. When the basal levels are then reduced from the values obtained after the addition of standard, the result is sometimes recoveries <100%. However the obtained recoveries indicate that the method is sufficiently accurate for the purpose. The plasma method is highly sensitive, which makes it possible to measure even low quantities of equol in plasma (<1 nmol/l). The three-fold concentration of the samples with a very low amount of equol, move the values to a more linear part of the standard curve, resulting in a better precision of the assay. The excellent correlation of the GC–MS method with the TR-FIA method for plasma indicates that the immunoassay method for equol in plasma gives the accurate biological information we need.

Although the urine TR-FIA method has a good correlation with the GC–MS method, there is a considerable overestimation of equol, which increases when the equol concentration decreases. The overestimation is probably due to the contribution of the matrix to the signal; the lower the concentration of equol, the higher the influence on the results. The interference of structurally similar compounds can also influence the overestimation. It is much more favorable to measure plasma than to determine urine equol because the plasma values are more stable and the method highly specific. Much work has been carried out to produce other equol antibodies more specific for the assay of urine samples, but without success. However, because of the good correlation with the GC–MS method the formula can be used for correction of urine values leading to good results when concentrations are >20 nmol/l. For lower values we do not recommend the use of our TR-FIA method.

In conclusion, the TR-FIA method developed is convenient, precise and sensitive and highly specific for plasma samples. For urine samples the values have to be corrected by a formula obtained by comparing the TR-FIA results with those obtained with a reference GC–MS method. The incubation time is short, and a DELFIA automated assay measuring 96 samples can be completed in 4 h. The most time consuming steps are the hydrolysis for the urine method and the hydrolysis and extraction for the plasma method. From 20 μl urine and 200 μl plasma after hydrolysis (and extraction), three–four compounds can easily be measured. TR-FIA methods now exist for enterolactone, genistein, daidzein and *O*-desmethylangolensin. Further research is now done to evaluate methods without extraction or even without hydrolysis of the conjugated compounds. The method has now also been successfully applied, including some additional steps, to the analysis of 50–100 mg brain samples [78].

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References

[1] H. Adlercreutz, W. Mazur, Phyto-oestrogens and Western diseases, Ann. Med. 29 (1997) 95–120.

[2] M. Vanharanta, S. Voutilainen, T.A. Lakka, M. van der Lee, H. Adlercreutz, J.T. Salonen, Risk of acute coronary events according to serum concentrations of enterolactone: a prospective population-based case–control study, Lancet 354 (1999) 2112–2115.

[3] H. Adlercreutz, Phyto-oestrogens and cancer, Lancet Oncol. 3 (2002) 364–373.

[4] R.B. Bradbury, D.E. White, Estrogens and related substances in plants, in: R.S. Haaris, G.F. Marrian, K.V. Thimann (Eds.), Vitamins and Hormones. Advances in Research and Applications, Academic Press, New York, 1954, pp. 207–233.

[5] A. Eldridge, W.F. Kwolek, Soybean isoflavones: effect of environment and variety on composition, J. Agric. Food Chem. 31 (1983) 394–396.

[6] K.R. Price, G.R. Fenwick, Naturally occurring oestrogens in foods—a review, Food Addit. Contam. 2 (1985) 73–106.

[7] J.M. Hodgson, K.D. Croft, I.B. Puddey, T.A. Mori, L.J. Beilin, Soybean isoflavonoids and their metabolic products inhibit in vitro lipoprotein oxidation in serum, J. Nutr. Biochem. 7 (1996) 664–669.

[8] Y. Folman, G.S. Pope, The interaction in the immature mouse of potent oestrogens with coumestrol, genistein and other utero-vaginotrophic compounds of low potency, J. Endocrinol. 34 (1966) 215–225.

[9] Y. Folman, G.S. Pope, Effect of norethisterone acetate, dimethylstilboestrol, genistein and coumestrol on uptake of [^{3}H]oestradiol by uterus, vagina and skeletal muscle of immature mice, J. Endocrinol. 44 (1969) 213–218.

[10] B.Y. Tang, N.R. Adams, Effect of equol on oestrogen receptors and on synthesis of DNA and protein in the immature rat uterus, J. Endocrinol. 85 (1980) 291–297.

[11] W.D. Kitts, F.E. Newsome, V.C. Runeckles, The estrogenic and antiestrogenic effects of coumestrol and zearalanol on the immature rat uterus, Can. J. Anim. Sci. 63 (1983) 823–834.

[12] P.L. Whitten, F. Naftolin, Dietary estrogens—a biologically active background for estrogen action. In: R.B. Hochberg, F. Naftolin (Eds.), New Biology of Steroid Hormones, Raven Press, New York, 1991, pp. 155–167.

[13] H. Adlercreutz, C. Bannwart, K. Wähälä, T. Mäkelä, G. Brunow, T. Hase, P.J. Arosemena, J.T. Kellis Jr., L.E. Vickery, Inhibition of human aromatase by mammalian lignans and isoflavonoid phytoestrogens, J. Steroid Biochem. Mol. Biol. 44 (1993) 147–153.

[14] Y. Mousavi, H. Adlercreutz, Genistein is an effective stimulator of SHBG production in Hep-G2 human liver cancer cells and suppresses proliferation of these cells in culture, Steroids 58 (1993) 301–304.

[15] S.I. Mäkelä, L.H. Pylkkänen, R.S.S. Santti, H. Adlercreutz, Dietary soybean may be antiestrogenic in male mice, J. Nutr. 125 (1995) 437–445.

[16] E. Monti, B.K. Sinha, Antiproliferative effect of genistein and adriamycin against estrogen-dependent and -independent human breast carcinoma cell lines, Anticancer Res. 14 (1994) 1221–1226.

[17] T. Hirano, M. Gotoh, K. Oka, Natural flavonoids and lignans are potent cytostatic agents against human leukemic HL-60 cells, Life Sci. 55 (1994) 1061–1069.

[18] L. Schweigerer, K. Christeleit, G. Fleischmann, H. Adlercreutz, K. Wahala, T. Hase, M. Schwab, R. Ludwig, T. Fotsis, Identification in human urine of a natural growth inhibitor for cells derived from solid paediatric tumours, Eur. J. Clin. Invest. 22 (1992) 260–264.

[19] A. Constantinou, K. Kiguchi, E. Huberman, Induction of differentiation and DNA strand breakage in human HL-60 and K-562 leukemia cells by genistein, Cancer Res. 50 (1990) 2618–2624.

[20] T. Fotsis, M. Pepper, H. Adlercreutz, G. Fleischmann, T. Hase, R. Montesano, L. Schweigerer, Genistein, a dietary-derived inhibitor of in vitro angiogenesis, Proc. Natl. Acad. Sci. U.S.A. 90 (1993) 2690–2694.

[21] H. Adlercreutz, Human health and phytoestrogens, in: K.S. Korach (Ed.), Reproductive and Developmental Toxicology, Marcel Dekker, New York, 1998, pp. 299–371.

[22] D.A. Shutt, R.H. Weston, J.P. Hogan, Quantitative aspects of phyto-oestrogen metabolism in sheep fed on subterranean clover (*Trifolium subterraneum* cultivar *clare*) or red clover (*Trifolium pratense*), Aust. J. Agric. Res. 21 (1970) 713–722.

[23] S. Heinonen, K. Wähälä, H. Adlercreutz, Identification of isoflavone metabolites dihydrodaidzein, dihydrogenistein, 6′-hydroxy-*O*-desmethylangolensin and *cis*-4-hydroxy-equol and a new isoflavone, 4′,7,8-trihydroxyisoflavone, from human urine by GC–MS with the authentic reference standards, Anal. Biochem. 274 (1999) 211–219.

[24] K.D.R. Setchell, H. Adlercreutz, Mammalian lignans and phyto-oestrogens. Recent studies on their formation, metabolism and biological role in health and disease, in: I. Rowland (Ed.), Role of the Gut Flora in Toxicity and Cancer, Academic Press, London, 1988, pp. 315–345.

[25] G.E. Kelly, C. Nelson, M.A. Waring, G.E. Joannou, A.Y. Reeder, Metabolites of dietary (soya) isoflavones in human urine, Clin. Chim. Acta 223 (1993) 9–22.

[26] I. Rowland, H. Wiseman, T. Sanders, H. Adlercreutz, E. Bowey, Metabolism of oestrogens and phytoestrogens: role of the gut microflora, Biochem. Soc. Trans. 27 (1999) 304–308.

[27] A.W.H. Braden, N.K. Hart, J.A. Lamberton, The oestrogenic activity of and metabolism of certain isoflavones in sheep, Aust. J. Agric. Res. 18 (1967) 335–348.

[28] T. Yasuda, J. Ueda, K. Ohsawa, Urinary metabolites of genistein administered orally to rats, Chem. Pharm. Bull. Tokyo 49 (2001) 1495–1497.

[29] K.D.R. Setchell, S.P. Borriello, P. Hulme, M. Axelson, Nonsteroidal estrogens of dietary origin: possible roles in hormone-dependent disease, Am. J. Clin. Nutr. 40 (1984) 569–578.

[30] H. Adlercreutz, H. Honjo, A. Higashi, T. Fotsis, E. Hämäläinen, T. Hasegawa, H. Okada, Urinary excretion of lignans and isoflavonoid phytoestrogens in Japanese men and women consuming traditional Japanese diet, Am. J. Clin. Nutr. 54 (1991) 1093–1100.

[31] G.E. Kelly, G.E. Joannou, A.Y. Reeder, C. Nelson, M.A. Waring, The variable metabolic response to dietary isoflavones in humans, Proc. Soc. Exp. Biol. Med. 208 (1995) 40–43.

[32] I.R. Rowland, H. Wiseman, T.A.B. Sanders, H. Adlercreutz, E.A. Bowey, Interindividual variation in metabolism of soy isoflavones and lignans: influence of habitual diet on equol production by the gut microflora, Nutr. Cancer 36 (2000) 27–32.

[33] J.W. Lampe, S.C. Karr, A.M. Hutchins, J.L. Slavin, Urinary equol excretion with a soy challenge: influence of habitual diet, Proc. Soc. Exp. Biol. Med. 217 (1998) 335–339.

[34] H. Adlercreutz, T. Fotsis, C. Bannwart, T. Mäkelä, K. Wähälä, G. Brunow, T. Hase, Assay of lignans and phytoestrogens in urine of women and in cow milk by GC/MS (SIM), in: J.F.J. Todd (Ed.), Advances in Mass Spectrometry, Proceedings of the 10th International Mass Spectrometry Conference, Wiley, Chichester, Sussex, 1986, pp. 661–662.

[35] D.R. Lindsay, R.W. Kelly, The metabolism of phyto-oestrogens in sheep, Aust. Vet. J. 46 (1970) 219–222.

[36] M. Axelson, D.N. Kirk, R.D. Farrant, G. Cooley, A.M. Lawson, K.D.R. Setchell, The identification of the weak oestrogen equol [7-hydroxy-3-(4′-hydroxyphenyl)chroman] in human urine, Biochem. J. 201 (1982) 353–357.

[37] H. Adlercreutz, T. Fotsis, R. Heikkinen, J.T. Dwyer, M. Woods, B.R. Goldin, S.L. Gorbach, Excretion of the lignans enterolactone and enterodiol and of equol in omnivorous and vegetarian women and in women with breast cancer, Lancet 2 (1982) 1295–1299.

[38] N. Sathyamoorthy, T.T.Y. Wang, Differential effects of dietary phyto-oestrogens daidzein and equol on human breast cancer MCF-7 cells, Eur. J. Cancer 33 (1997) 2384–2389.

[39] L. Markiewicz, J. Garey, H. Adlercreutz, E. Gurpide, In vitro bioassays of non-steroidal phytoestrogens, J. Steroid Biochem. Mol. Biol. 45 (1993) 399–405.

[40] A. Arora, T.M. Byre, M.G. Nair, G.M. Strasburg, Modulation of liposomal membrane fluidity by flavonoids and isoflavonoids, Arch. Biochem. Biophys. 373 (2000) 102–109.

[41] D. Ingram, K. Sanders, M. Kolybaba, D. Lopez, Case–control study of phyto-oestrogens and breast cancer, Lancet 350 (1997) 990–994.

[42] A.M. Duncan, B.E. Merz-Demlow, X. Xu, W.R. Phipps, M.S. Kurzer, Premenopausal equol excretors show plasma hormone profiles associated with lowered risk of breast cancer, Cancer Epidemiol. Biomarkers Prev. 9 (2000) 581–586.

[43] H. Adlercreutz, H. Markkanen, S. Watanabe, Plasma concentrations of phyto-oestrogens in Japanese men, Lancet 342 (1993) 1209–1210.

[44] H. Adlercreutz, T. Fotsis, C. Bannwart, K. Wähälä, G. Brunow, T. Hase, Isotope dilution gas chromatographic–mass spectrometric method for the determination of lignans and isoflavonoids in human urine, including identification of genistein, Clin. Chim. Acta 199 (1991) 263–278.

[45] G.E. Joannou, G.E. Kelly, A.Y. Reeder, M. Waring, C. Nelson, A urinary profile study of dietary phytoestrogens. The identification and mode of metabolism of new isoflavonoids, J. Steroid Biochem. Mol. Biol. 54 (1995) 167–184.

[46] K.D.R. Setchell, L. Zimmer-Nechemias, J.N. Cai, J.E. Heubi, Exposure of infants to phyto-oestrogens from soy-based infant formula, Lancet 350 (1997) 23–27.

[47] H. Adlercreutz, T. Fotsis, J. Lampe, K. Wähälä, T. Mäkelä, G. Brunow, T. Hase, Quantitative determination of lignans and isoflavonoids in plasma of omnivorous and vegetarian women by isotope dilution gas-chromatography mass-spectrometry, Scand. J. Clin. Lab. Invest. 53 (1993) 5–18.

[48] H. Adlercreutz, T. Fotsis, S. Watanabe, J. Lampe, K. Wähälä, T. Mäkelä, T. Hase, Determination of lignans and isoflavonoids in plasma by isotope dilution gas chromatography–mass spectrometry, Cancer Detect. Prev. 18 (1994) 259–271.

[49] A.A. Franke, L.J. Custer, High-performance liquid chromatographic assay of isoflavonoids and coumestrol from human urine, J. Chromatogr. B Biol. Med. Appl. 662 (1994) 47–60.

[50] X. Xu, H.J. Wang, P.A. Murphy, L. Cook, S. Hendrich, Daidzein is a more bioavailable soymilk isoflavone than is genistein in adult women, J. Nutr. 124 (1994) 825–832.

[51] T. Nurmi, H. Adlercreutz, Sensitive HPLC method for profiling plasma phytoestrogens using coulometric electrode array detection, Anal. Biochem. 274 (1999) 110–117.

[52] S. Bauminger, H.R. Lindner, E. Perel, R. Arnon, Antibodies to a phyto-oestrogen: antigenicity of genistein coupled to a synthetic polypeptide, J. Endocrinol. 44 (1969) 567–578.

[53] W.Q. Wang, Y. Tanaka, Z.K. Han, J. Cheng, Radioimmunoassay for quantitative analysis of formononetin in blood plasma and rumen fluid of wethers fed red clover, J. Agric. Food Chem. 42 (1994) 1584–1587.

[54] O. Lapcík, R. Hampl, N. Al-Maharik, A. Salakka, K. Wähälä, H. Adlercreutz, A novel radioimmunoassay for daidzein, Steroids 62 (1997) 315–320.

[55] O. Lapcík, R. Hampl, M. Hill, K. Wähälä, N. Al-Maharik, H. Adlercreutz, Radioimmunoassay of free genistein in human serum, J. Steroid Biochem. Mol. Biol. 64 (1998) 261–268.

[56] F. Kohen, S. Lichter, B. Gayer, J. Deboever, L.J.W. Lu, The measurement of the isoflavone daidzein by time resolved fluorescent immunoassay: a method for assessment of dietary soya exposure, J. Steroid Biochem. Mol. Biol. 64 (1998) 217–222.

[57] H. Adlercreutz, G.J. Wang, O. Lapcík, R. Hampl, K. Wähälä, T. Mäkelä, K. Lusa, M. Talme, H. Mikola, Time-resolved fluoroimmunoassay for plasma enterolactone, Anal. Biochem. 265 (1998) 208–215.

[58] G.J. Wang, O. Lapcík, R. Hampl, M. Uehara, N. Al-Maharik, K. Stumpf, H. Mikola, K. Wähälä, H. Adlercreutz, Time-resolved fluoroimmunoassay of plasma daidzein and genistein, Steroids 65 (2000) 339–348.

[59] M. Uehara, O. Lapcík, R. Hampl, N. Al-Maharik, T. Mäkelä, K. Wähälä, H. Mikola, H. Adlercreutz, Rapid analysis of phytoestrogens in human urine by time-resolved fluoroimmunoassay, J. Steroid Biochem. Mol. Biol. 72 (2000) 273–282.

[60] T. Fotsis, H. Adlercreutz, The multicomponent analysis of estrogens in urine by ion exchange chromatography and GC–MS. I. Quantitation of estrogens after initial hydrolysis of conjugates, J. Steroid Biochem. 28 (1987) 203–213.

[61] H. Adlercreutz, P.I. Musey, T. Fotsis, C. Bannwart, K. Wähälä, T. Mäkelä, G. Brunow, T. Hase, Identification of lignans and phytoestrogens in urine of chimpanzes, Clin. Chim. Acta 158 (1986) 147–154.

[62] K. Wähälä, T.A. Hase, Expedient synthesis of polyhydroxyisoflavones, J. Chem. Soc. Perkin. Trans. 1 (1991) 3005–3008.

[63] K. Wähälä, T. Hase, H. Adlercreutz, Synthesis and labeling of isoflavone phytoestrogens, including daidzein and genistein, Proc. Soc. Exp. Biol. Med. 208 (1995) 27–32.

[64] K. Wähälä, A. Salakka, H. Adlercreutz, Synthesis of novel mammalian metabolites of the isoflavonoid phytoestrogens daidzein and genistein, Proc. Soc. Exp. Biol. Med. 217 (1998) 293–299.

[65] C. Bannwart, H. Adlercreutz, T. Fotsis, K. Wähälä, T. Hase, G. Brunow, Identification of *O*-desmethylangolensin, a metabolite of daidzein, and of matairesinol, one likely plant precursor of the animal lignan enterolactone, in human urine, Finn. Chem. Lett. (1984) 120–125.

[66] A. Salakka, Synthesis of isoflavonoid metabolites, PhD thesis, University of Helsinki, ISBN 952-91-4027-4, Saarijärvi, Finland, Gummerus Printing, 2001, pp. 1–81.

[67] K. Wähälä, J. Koskimies, M. Mesilaakso, A. Salakka, T. Leino, H. Adlercreutz, The synthesis, structure and anticancer activity of *cis*- and *trans*-4′,7-dihydroxyisoflavan-4-ols, J. Org. Chem. 62 (1997) 7690–7693.

[68] T. Jokela, K. Wähälä, Optimized synthesis of isoflavans by catalytic hydrogenation, unpublished data.

[69] A. Hoikkala, S.M. Heinonen, K. Wähälä, Preparation of new nutraceutical isoflavones and their mammalian metabolites, in: Proceedings of the Fourth International Symposium on the Role of Soy in Preventing and Treating Chronic Diseases, Hyatt Islandia, San Diego, California, November 4–7, 2001.

[70] N. Al-Maharik, S. Kaltia, K. Wähälä, Regioselective mono-*O*-carboxymethylation of polyhydroxyisoflavones, Mol. Online 3 (1999) 20–24.

[71] N. Al-Maharik, Synthesis of isoflavonoid derivatives for immunoassay, PhD thesis, University of Helsinki, ISBN 952-91-1738-8, Vantaa, Finland, Tummavuoren kirjapaino Oy, 2000, pp. 1–63.

[72] E.A. Yatsimirskaya, E.M. Gavrilova, A.M. Egorov, A. Levashov, Preparation of conjugates of progesterone with bovine serum albumin in the reversed micellar medium, Steroids 58 (1993) 547–550.

[73] B. Cook, G.H. Beastall, Measurement of steroid hormone concentrations in blood, urine and tissues, in: B. Green, R.E. Leake (Eds.), Steroid Hormones, A Practical Approach, IRL Press Ltd., Oxford, 1987, pp. 1–65.

[74] V.M. Mukkala, H. Mikola, I. Hemmilä, The synthesis and use of activated *N*-benzyl derivatives of diethylenetriaminetetraacetic acid: alternative reagents for labeling of antibodies with metal ions, Anal. Biochem. 176 (1989) 319–325.

[75] H. Mikola, P. Miettinen, Preparation of europium labeled derivatives of cortisol for time-resolved fluoroimmunoassay, Steroids 56 (1991) 17–21.

[76] A.P. Wilkinson, P.I. Creeke, H.A. Lee, M.J.C. Rhodes, M.R.A. Morgan, Measurement of daidzein and equol in human serum by enzyme-linked immunosorbent assay (ELISA), in: S.

Bausch-Goldbohm, A. Kardinaal, F. Serra (Eds.), Bioactive Plant Cell Wall Components in Nutrition and Health. Phytoestrogens: Exposure, Bioavailability, Health Benefits and Safety Concerns, European Communities, Doorweth, The Netherlands, 1999, pp. 85–88.

[77] C. Bennetau-Pelissero, C. LeHouerou, F. Lemenn, B. Bennetau, ELISAs for genistein and daidzein, in: S. Bausch-Goldbohm, A. Kardinaal, F. Serra (Eds.), Bioactive Plant Cell Wall Components in Nutrition and Health. Phytoestrogens: Exposure, Bioavailability, Health Benefits and Safety Concerns, European Communities, Doorweth, The Netherlands, 1999, pp. 85–88.

[78] E.D. Lephart, H. Adlercreutz, T.D. Lund, Dietary soy phytoestrogen effects on brain structure and aromatase in Long-Evans rats, NeuroReport 12 (2001) 3451–3455.