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Rapid analysis of phytoestrogens in human urine by time-resolved fluoroimmunoassay

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Abstract

A time-resolved fluoroimmunoassay (TR-FIA), with europium labeled phytoestrogens as tracers, was developed for the quantitative determination of enterolactone, genistein and daidzein in human urine. The aim was to create a method for the screening of large populations in order to assess the possible correlations between the urinary levels and the risk of Western diseases.

After the synthesis of the 5′-carboxymethoxy derivative of enterolactone and 4′-*O*-carboxymethyl derivatives of daidzein and genistein, the respective compound was coupled to bovine serum albumin and then used as an antigen in the immunization of rabbits. The same derivatives of the phytoestrogen were used in preparing the europium tracers. After the enzymatic hydrolysis, the TR-FIA was carried out using the Victor 1420 multilabel counter. The method has sufficient sensitivity to measure the phytoestrogens at concentrations even below 5 nmol/l. The intra- and inter-assay coefficients of variation, at three different concentrations, varied from 1.9 to 5.3 and from 2.4 to 9.7, respectively.

We measured urinary enterolactone, genistein and daidzein in 215 samples from Finnish healthy women and found that more than 50% of the values ranged between 1 and 7, <0.1 and 0.6 and below 0.6 μmol/24 h, respectively. The TR-FIA method including only a hydrolysis step gave higher values than those measured by gas chromatography–mass spectrometry (GC–MS). However, the assay results by the present method showed strong correlation with those obtained by GC–MS. It is concluded that the TR-FIA is suitable for population screening of urinary phytoestrogens.

Keywords: Immunoassay; Phytoestrogens; Isoflavones; Lignans; Urine; Enterolactone; Daidzein; Genistein; Vegetarian

1. Introduction

About 20 years ago two groups of hormone-like compounds, the isoflavonoids and lignans, were found in human urine and later in plasma and other biological fluids [1–8]. The isoflavones were well-known in the veterinary field [9], but completely unknown in human medicine. These compounds have after that received increasing attention as phytoestrogens because of their estrogen-like biological properties and pharmacological interaction with various enzymes and proteins. These activities may result in protection against many Wes-

Abbreviations: GC, gas–liquid chromatography; HPLC, high performance liquid chromatography; MS, mass spectrometry; RIA, radioimmunoassay; TR-FIA, time-resolved fluoroimmunoassay; EIA, enzymeimmunoassay; FIA, fluoroimmunoassay; BSA, bovine serum albumin; 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_0, binding of tracer to antibody in the absence of unlabeled analyte; *T*, total amount of tracer added.

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tern diseases [10–13]. The highest phytoestrogen consumption and concentrations in urine and plasma are found in subjects living in countries with low cancer and coronary heart disease incidence, and the lowest values were found in breast cancer patients or in women at high risk for breast cancer [5,14–18].

Isoflavones in human urine, occurring mainly as glucuronide and sulfate conjugates [19], have been analyzed after hydrolysis and extraction practically exclusively by gas chromatography coupled with mass spectrometry (GC–MS) [20–23] or by high performance liquid chromatography (HPLC) [24,25]. Until now approximately 15 lignans and isoflavonoids have been identified in human urine [12], and recently we have detected many more metabolites (unpublished). Usually 5–8 metabolites are analyzed with these methods, and isotope dilution GC–MS in the selected ion monitoring mode (ID-GC–MS-SIM) also allows assays at the low levels found in plasma [26]. This methodology is very sensitive, but requires many preparative and column chromatographic steps. These are time consuming. The instrument is expensive and needs considerable experience. Therefore, ID-GC–MS-SIM is not suitable for screening purposes in large populations, but is excellent as a reference method.

Recently, sensitive radioimmunoassay (RIA) methods have been developed to measure unconjugated formononetin in plasma [27], and total and unconjugated daidzein and genistein in human biological fluids [28,29]. This is usually the first step before development of non-radioisotopic assays with more stable tracers and no waste problems. We recently developed more simple and rapid procedures to analyze hydrolyzed and extracted phytoestrogens in plasma by TR-FIA [30–32]. The speed, convenience, precision and reliability are advantages of this method for clinical utility and epidemiological surveys. A TR-FIA for urinary daidzein has recently been developed by Kohen et al. [33].

In the present study we present a TR-FIA method for the simultaneous determination of three phytoestrogens, enterolactone, daidzein, and genistein, in human urine. The values were compared with those measured by the GC–MS reference method. Furthermore, this method was applied to the analysis of urinary phytoestrogens in healthy Finnish women.

2. Materials and methods

2.1. Chemicals

Enterolactone, enterodiol, matairesinol, daidzein, genistein and equol were synthesized as previously described for analysis of foods or biological fluids [8,34,35]. Secolariciresinol was obtained by hydride reduction of matairesinol and was further converted to anhydrosecoisolariciresinol by perchloroacid treatment [36]. BSA, diethyl ether, methanol, and ethanol were of analytical grade (Merck AG, Darmstadt, Germany). β-glucuronidase (EC 3.2.1.31) (Boehringer, Mannheim, Germany, catalogue No. 1585665), and DCC and NHS were from Sigma (St Louis, USA), as well as sulfatase (EC 3.1.6.1) (catalogue No. S-9626).

The assay buffer was 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 l and was used for dilution of antiserum and Eu-labeled phytoestrogen derivatives. Acetate buffer 0.1 mol/l, pH 5.0, was used in enzyme hydrolysis of the three phytoestrogen conjugates (enterolactone, genistein, daidzein). The stock solutions of standards were made in ethanol or by first dissolving the standards in a small amount of dimethylsulfoxide (DMSO).

2.2. Synthesis of 5′-carboxymethoxyenterolactone, 4′-O-carboxymethylgenistein, and 4′-O-carboxymethyldaidzein

The dibenzylbutyrolactone framework of the enterolactone acid derivative (5′-carboxymethoxyenterolactone) was prepared by the tandem conjugate addition method from 3,5-dihydroxybenzoic acid [32,34]. The acid side chain was then added as an ester by selectively alkylating the phenolic hydroxy group at the position 5′ in the aromatic ring. After removal of the protection groups the ester was hydrolyzed under basic conditions to the desired acid.

4′-*O*-carboxymethylgenistein or -daidzein was prepared by selective alkylation of the phenolic 4′-hydroxy group of genistein or daidzein using potassium-*tert*-butoxide and ethyl bromoacetate in dimethylformamide, respectively. After the alkylation the ethyl ester was hydrolyzed under acidic conditions to the desired acid in good yield.

2.3. Immunogen synthesis and immunization

The immunogen was synthesized according to Yatsimirskaya et al. [37] with minor modifications. In brief: 12 mg of 5′-carboxymethoxyenterolactone or 5.1 mg of 4′-*O*-carboxymethylgenistein or -daidzein was left to react overnight with DDC and NHS (molar ratio 3:4:5) in 200 μl of anhydrous dimethylformamide. On the next 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 between the activated compounds and BSA was 70:1. BSA (29 mg) was dissolved in 1.5 ml of 0.02 M bicarbonate buffer, pH 8.5. This solution was added dropwise to 10 ml of 0.3 M dioctyl sulfosuccinate in octane

under continuous stirring. After the mixture became clear, a dimethylformamide solution of the activated intermediate formed from respective carboxyderivatives of enterolactone, daidzein or genistein was added. The mixture was stirred for an additional 24 h at ambient temperature. Enterolactone, genistein or daidzein-BSA conjugates were isolated from mixtures by precipitation with three volumes 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 0.22 μm Millipore filter and lyophilized. The hapten/carrier protein ratio of the conjugates was estimated by titration of free NH_2 groups by trinitrobenzenesulfonic acid [38], yielding 15–17 molecules of the hapten per one molecule of BSA. Rabbits were immunized and antisera collected using a standard procedure [39].

2.4. Labeling of enterolactone, genistein and daidzein derivatives with europium chelate

5′-carboxymethoxyenterolactone, genistein or daidzein 4′-*O*-(carboxymethyl) ether was labeled in 0.5 M 4-morpholinoethanesulfonic acid buffer, pH 5.5, with 4-aminobenzyldiethylenetriaminetetraacetic acid europium chelate [40] using 1-(3-(dimethylamino)propyl)ethyl carbodiimide hydrochloride as a condensation agent, and purified on a preparative TLC plate, as described previously for steroid derivatives [41]. The labeled enterolactone, genistein or daidzein derivative was further purified by HPLC (Gradient Pump 2249, UV-monitor UvicordSII, and Superdex Peptide column: eluent 10 or 20% acetonitrile in 0.05 mol/l NaCl with 0.05 mol/l Tris-HCl, pH 7.5) from Pharmacia (Uppsala, Sweden).

2.5. Determination of enterolactone, genistein and daidzein by GC–MS

ID-GC–MS-SIM with synthesized deuterated internal standard was used as a reference method. The method is very complicated, because it also includes the determination of 14 estrogens. It has been described in detail including results of the validation studies in Refs. [21,42].

2.6. Pretreatment of urine sample

One ml of 0.1 M acetate buffer (pH 5), containing 0.2 U/ml of β-glucuronidase and 2 U/ml of sulfatase (hydrolysis reagent) was added to plastic tubes with 50 μl of urine. They were mixed and incubated overnight at 37°C, and 20 μl of the solution for each compound to be assayed was subjected to TR-FIA. When isoflavone concentration is very low the analysis may start from 100 to 200 μl of urine.

2.7. Time-resolved fluoroimmunoassay

Twenty μl of standard or hydrolyzed extract in a buffer was pipetted into prewashed goat anti-rabbit IgG coated microtitration wells, 100 μl of diluted antiserum (dilution 1:250,000, 1:50, 000 or 1:40,000, for enterolactone, genistein and daidzein, respectively) in 0.5% BSA Tris-HCl buffer (50 mmol/l, pH 7.8) and 100 μl of the tracer, diluted to suitable concentration, was added to each well. After incubation and shaking the strips slowly on a DELFIA plate shaker at room temperature for 90 min, the strips were washed using a DELFIA plate washer (using the No. 29 T3 program). Enhancement solution (200 μl) was added to each well and the strips were shaken slowly for an additional 5 min. The enhanced fluorescences were measured in a VICTOR 1420 multilabel counter. Calculation of the final result was done according to the formula:

$$\text{Final results} = \text{Concentration (read)} \times \text{dilution factor}$$

(nmol/l).

2.8. Samples and treatments

Twenty-five 24-h urine samples obtained from healthy postmenopausal women were used for calculating the correlation coefficient between the results obtained with the TR-FIA and GC–MS methods. Other urine samples (mostly 72-h collections) ($n = 215$, 126 from omnivores and 89 from vegetarians) were obtained from healthy Finnish women for calculation of the frequency distribution of values in the population in the Helsinki area. The collection bottles contained 1.5 g ascorbic acid and 0.001 mol/l NaN_3 (added after collection), and had been stored for many years at −20°C until the assay.

2.9. Statistical treatment

Values obtained by the TR-FIA method were compared with those obtained by the GC–MS method and correlation coefficients calculated between the results using an Excel program Version 5.0 or a Cricket Graph program 1.3.1 for Macintosh. Statistical analysis was also done by the SPSS package program Version 6.1J on Windows 95. Differences were considered significant at $p < 0.05$ for homogeneity and Student's *t*-test.

Table 1
Optimal conditions for the phytoestrogen assays

Phytoestrogen	Antibody dilution	Eu-labeled conjugate dilutation	B_0/T (%)
Enterolactone	1:250,000	1:400,000	16
Genistein	1:50,000	1:400,000	27
Daidzein	1:40,000	1:40,000	15

3. Results

3.1. Immunoassay optimization

Various concentrations of antibody and conjugate were tested to get an optimized reaction by the assay (Table 1). The non-specific binding of the tracer without the antibody was less than 1.5% of the B_0 value. We also investigated the effect of incubation time on the immunoreaction and selected 90-min for incubation. By this time the maximum fluorescence signal reached a plateau.

3.2. Precision

Intra- and inter-assay coefficients of variation were assessed by measuring enterolactone, genistein, and daidzein in three samples of hydrolyzed urine with different concentrations. The results are shown in Table 2. The intra- and inter-assay CVs ranged from 1.9 to 5.3% and from 2.4 to 9.7%, respectively.

3.3. Sensitivity

Sensitivity, expressed as the minimal amount of enterolactone, genistein and daidzein distinguishable from the zero sample with 95% probability, was around 2 pg/20 μl. The working range for the assay for enterolactone, genistein, and daidzein varied from 8.9 to 3218, 9.18 to 1998 or 5.08 to 1097.28 pg/20 μl, respectively, corresponding to urinary levels of 1.5–540, 1.7–370 or 1–216 nmol/l, respectively. Urines with higher concentrations had to be diluted before TR-FIA.

3.4. Specificity

Cross reactivities of selected lignans, isoflavonoids and flavonoids with the enterolactone, daidzein and genistein antisera are shown in Table 3. The cross reaction percentages for the daidzein antiserum were 6.0% for daidzin, and 206% for its 4′-methyl ether derivative formononetin, 3.5% for biochanin A and 3.1% for dihydrodaidzein. The antiserum against genistein cross-reacted with biochanin A (500%), formononetin (44.4%), dihydrogenistein (11.3%), genistin (7.6%), and daidzein (2.5%). The enterolactone antiserum did not show any cross reaction with the lignans or other diphenols tested (Table 3).

3.5. Correlation with the GC–MS method

Both the TR-FIA and the GC–MS method measure the free and the conjugated phytoestrogens, and should, in principle, give similar values. However, because in the urine GC–MS method, no recovery correction is made during the first purification step (using first a Sep-Pak C18 column and then a DEAE Sephadex column in the acetate form) and for loses during hydrolysis, the GC–MS values should be about 10% lower than the immunoassay values. For daidzein, the mean values by TR-FIA (249 nmol/l) were similar

Table 2
Intra- and inter-assay coefficients of variation (CV) for three urinary phytoestrogens determined by TR-FIA

Phytoestrogen	Concentration (nmol/l)	Number of assays[a]	Intra-assay CV (%)	Inter-assay CV (%)
Enterolactone	526.7	10(8)	5.3	4.2
	1113.1	10(8)	4.5	6.4
	3802.8	10(8)	1.9	2.4
Genistein	144.1	10(8)	3.0	8.7
	345.9	10(8)	2.7	4.4
	1203.2	10(8)	3.2	9.7
Daidzein	137.6	10(8)	2.3	6.1
	397.3	10(8)	5.0	5.2
	2167.0	10(8)	2.4	7.4

[a] Numbers in parentheses are the number of assays for calculation of inter-assay CV%.

Table 3
Specificity of enterolactone, genistein and daidzein antisera

Compounds	Enterolactone antiserum	Genistein antiserum percent cross reaction	Daidzein antiserum
Enterolactone	100	nd[a]	nd
Enterodiol	0.28	nd	nd
Matairesinol	0	nd	nd
Anhydrosecoisolariciresinol	0	nd	nd
Secoisolariciresinol	0	nd	nd
Daidzein	0	2.5	100
Formononetin	nd	44.4	206
Biochanin	nd	500	3.5
Daidzin	nd	1.0	6.0
Dihydrodaidzein	nd	0.1	3.1
Genistein	0	100	1.1
Dihydrogenistein	nd	11.3	0
Genistin	0	7.6	0
Equol	0	0.1	0
O-Desmethylangolensin	nd	0	0
Luteolin	nd	0	0
Quercetin	nd	0	0

[a] Not determined.

with the values by GC–MS (218 nmol/l; $n = 25$). But, for enterolactone the mean values were approximately 30% higher in the TR-FIA (3.23 μmol/l; $n = 25$) than in the GC–MS (2.50 μmol/l; $n = 25$), and for genistein the difference was about 115% (TR-FIA 205 nmol/l and GC–MS 95.0 nmol/l; $n = 25$). Even if ether extraction was used, enterolactone and genistein showed high values compared with the values if only hydrolysis was employed (enterolactone: 4.66 versus 3.23 μmol/l, $n = 25$; genistein: 279 versus 205 nmol/l, $n = 25$); however, the difference was larger for enterolactone, but smaller for genistein. On the other hand, as shown in Figs. 1–3, highly significant correlations between the results obtained by the TR-FIA and GC–MS methods were found for all three phytoestrogens [enterolactone: $r = 0.870$ ($p < 0.001$; $n = 25$); genistein: $r = 0.880$ ($p < 0.001$; $n = 25$); daidzein: $r = 0.990$ ($p < 0.001$; $n = 25$)]. It was, therefore, decided not to use extraction in this rapid screening method. The values may always be converted to "GC–MS values" by the formulas in Figs. 1–3 if comparisons of the values with those obtained with the GC–MS methodology are needed, e.g. when comparing results of assays with TR-FIA with those in the literature published by us.

3.6. Urinary levels of phytoestrogens

Urinary phytoestrogens were analyzed, mostly in 72-h urinary collections, in altogether 215 samples from healthy Finnish women including both omnivores and vegetarians. The frequency distribution values are shown in Figs. 4–6. The majority of the enterolactone values were lower than 10 μmol/24 h; 21.4, 25.6 and 21.4% of the subjects were in the range between 1 and 3, 3 and 5 and 5 and 7 μmol/24 h, respectively. 24.2% of the values were above 7 μmol/24 h and 7.4% of subjects had very low values (below 1 μmol/24 h). Urinary genistein and daidzein values were very low in Finnish women, but the vegetarians showed higher values than the omnivores (Figs. 5 and 6 and Table 4).

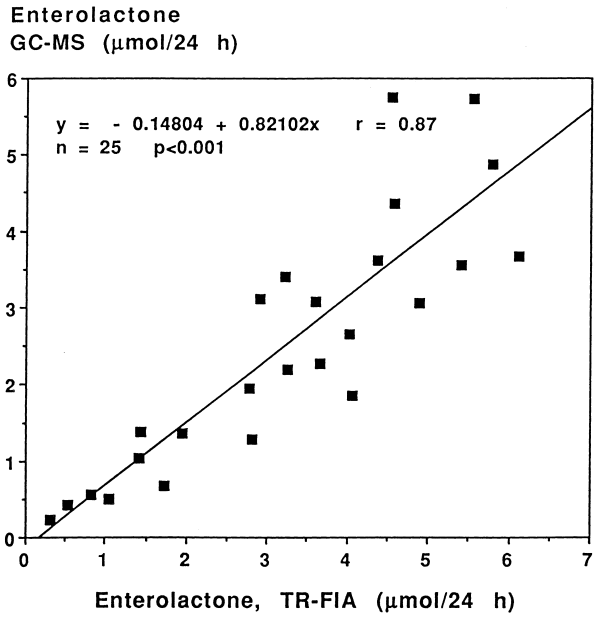

Fig. 1. Linear correlation between urinary enterolactone measured by combined gas chromatography–mass spectrometry in the selected ion monitoring mode and by time-resolved fluoroimmunoassay using europium labeled enterolactone derivative.

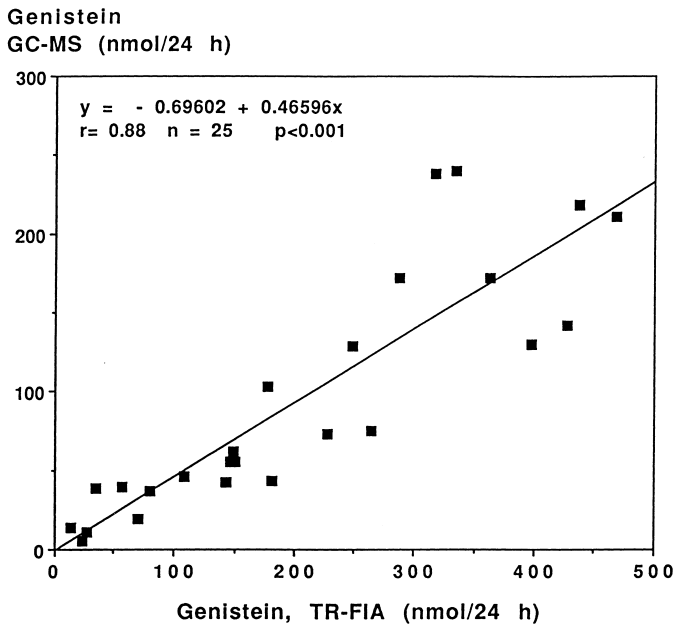

Fig. 2. Linear correlation between urinary genistein measured by combined gas chromatography–mass spectrometry in the selected ion monitoring mode and by time-resolved fluoroimmunoassay using europium labeled genistein derivative.

4. Discussion

There has been very few methods available for the assay of phytoestrogens in biological fluids using microsamples. The method for formononetin [27] was designed for studies in ruminants and is not suitable for human studies. A TR-FIA for urinary daidzein has recently been developed by Kohen et al. [33]. They used a monoclonal antibody against a seven-derivative of daidzein. We have a polyclonal antibody against 7-*O*-carboxymethylgenistein-BSA which in plasma radioimmunoassays has given similar results compared with the antibody to the 4′-*O*-carboxymethylgenistein which we have used in the present study [29]. Our antibody to the 7-*O*-derivative could probably have also been used for this study, as was found for the published radioimmunological procedure [29].

As a first step, we developed in collaborative work

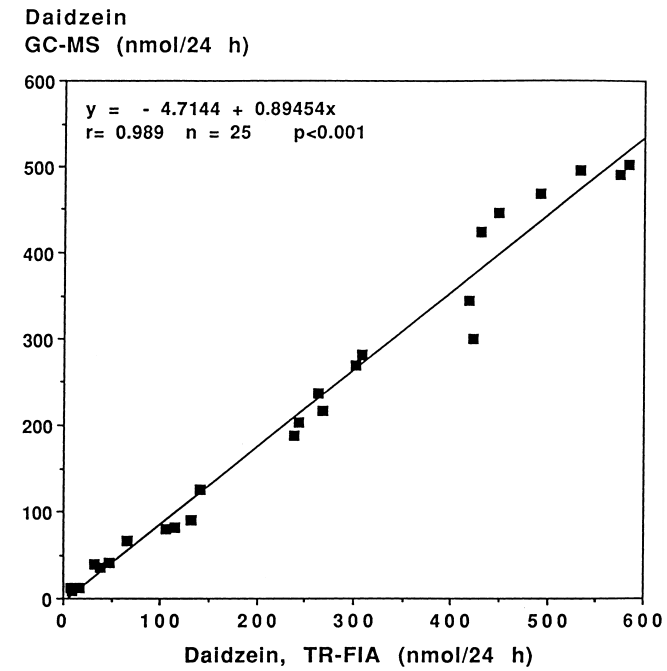

Fig. 3. Linear correlation between urinary daidzein measured by combined gas chromatography–mass spectrometry in the selected ion monitoring mode and by time-resolved fluoroimmunoassay using europium labeled daidzein derivative.

Table 4
The median, mean and standard error of mean (SEM) values of three urinary phytoestrogens in Finnish healthy women

	Omnivores (n = 126) μmol/l	Vegetarians (n = 89) μmol/l
Enterolactone		
Median	4.563	4.998
Mean	4.870	8.820[a]
SEM	0.299	1.396
Genistein		
Median	0.202	0.476
Mean	0.489	1.682[a]
SEM	0.112	0.271
Daidzein		
Median	0.137	0.494
Mean	0.555	2.123[a]
SEM	0.134	0.326

[a] Significantly higher than in omnivores, $p < 0.01$.

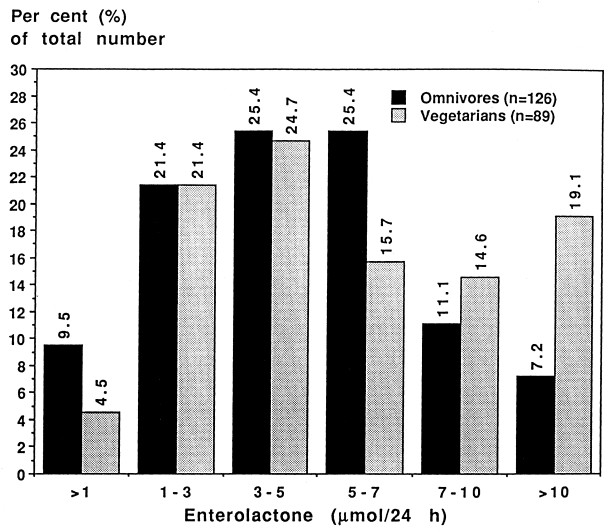

Fig. 4. Comparison of distribution of urinary enterolactone values of 126 omnivorous Finns and 89 vegetarian samples from subjects living in the urban Helsinki area.

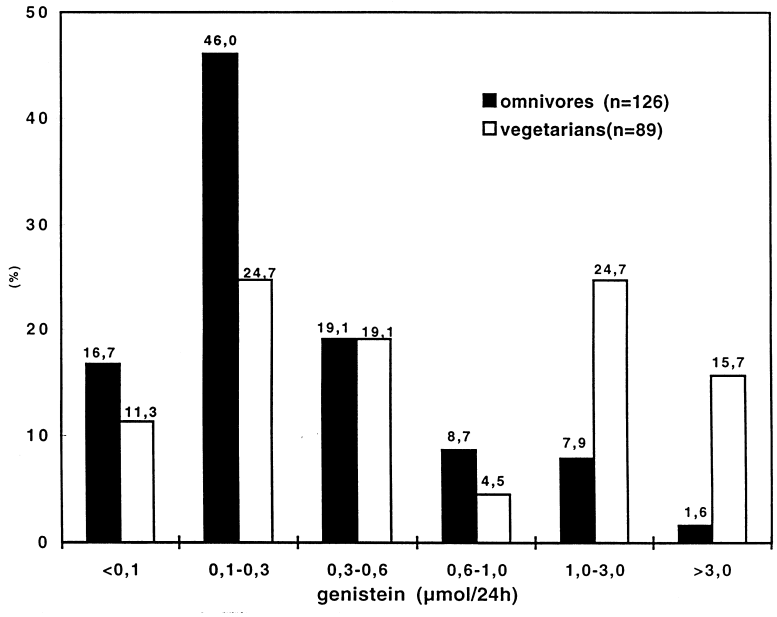

Fig. 5. Comparison of distribution of urinary genistein values of 126 omnivorous Finns and 89 vegetarian samples from subjects living in the urban Helsinki area.

radioimmunological methods for total and unconjugated daidzein and genistein in human biological fluids [28,29]. Because of the instability of the tracers and the waste problem the next step was to develop non-radioisotopic methods and because of its high sensitivity we have chosen the TR-FIA technique. Our antibodies were so good that we also recently developed more simple and rapid TR-FIA procedures for the analysis of hydrolyzed and extracted phytoestrogens in plasma [30–32]. We also have plasma methods for genistein and daidzein measuring only the unconjugated and 4′-conjugates by directly applying the final TR-FIA assay

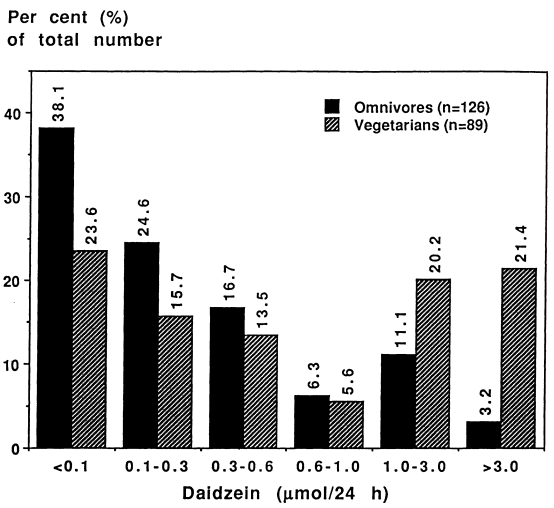

Fig. 6. Comparison of distribution of urinary daidzein values of 126 omnivorous Finns and 89 vegetarian samples from subjects living in the urban Helsinki area.

to 20 µl of plasma or serum. This is not possible for enterolactone because the antigen is produced with a derivative coupled to another site of the molecule than the available hydroxyl groups of enterolactone. However, these simplified techniques are being further evaluated before publication. The speed, convenience, precision and reliability are advantages of all these methods for use in clinical work and in epidemiological surveys.

It is obvious that the urinary TR-FIA method measures, in addition to the compound to be measured, some metabolites, with the exception of the daidzein assay. The overestimation of enterolactone is moderate, but for genistein it is high and if more specificity is needed in metabolic studies it would be necessary to include a chromatographic step. This overestimation occurs despite that the cross reactivities (Table 3) do not predict any overestimation for enterolactone and much less than the overestimation obtained for genistein. The same phenomenon has also been observed in steroid analyses when radioimmunoassay results have been compared with GC–MS results [43]. Because GC–MS is seldom used as a reference method for immunoassays the specificity of most radioimmunoassays has been based solely on the cross-reactivity studies. In our experience this frequently gives a wrong view of the specificity, because the interaction between many metabolites is not measured when only a single compound is studied in each cross reactivity experiment. We know that there is at least 13 metabolites of isoflavones in urine (unpublished), most of them previously unknown, but

their cross reactivities have not been studied because we have not yet synthesized all of them. Because the overestimation using the TR-FIA method is particularly prominent for genistein, we are going to raise new antibodies using a 2-carboxyderivative of genistein. It is of interest that for plasma samples using the same antisera, no overestimation is observed for genistein. For screening purposes the urinary method for genistein seems satisfactory because of the significant correlation ($p < 0.001$; $r = 0.88$) between the immunoassay and the GC–MS results. Because of the comparisons made it is simple to convert the values to "GC–MS results" (see formulas in Figs. 1–3). An advantage of this TR-FIA method is that it also measures the very low levels of isoflavones in subjects not consuming soy products.

Genistein cross-reacts highly with biochanin A. This isoflavone occurs in some legumes, particularly in chick peas (*Cicer arietinum*) about 1–3 mg/100 g and in red gram (*Cajanus cajan*) about 0.2 mg/100 g. High concentrations are seen in Kudzu (*Pueraria lobata*) leaves and Kudzu roots (1.2–1.4 mg/100 g) [44]. Because of its relatively rare occurrence biochanin A is seldom a problem, in addition, because it is converted to a great extent to genistein in the gut. Daidzein cross-reacts to a high degree with formononetin. Formononetin concentration is high in forage legumes like red clover (*Trifolium pratense*) (22 mg/100 g) and in Kudzu root (7 mg/100 g) and is much lower in soy beans (maximally 0.12 mg/100 g) and chick peas (0.1–0.2 mg/100 g) [44]. Formononetin is converted to daidzein in the gut and should not be a problem for the assay of daidzein if red clover is not consumed (e.g. in cattle samples after the cattle have grassed clover).

The values for genistein and daidzein in the Finnish population are very low, but significantly higher in the vegetarians compared to the omnivores (Table 4) in accordance with our early observations [20]. The enterolactone results are clearly higher than those we have obtained by GC–MS. This is partly due to the 30% higher values obtained by the TR-FIA method compared to the values obtained by GC–MS, but it is also due to the use of geometric means in the earlier study leading to lower mean values. Fiber intake correlates with urinary lignan excretion [10]. Figs. 4–6 suggest that the intake of fiber of the Finnish vegetarian women does not differ very much from the fiber intake of the omnivorous women. There are fewer vegetarians in the group with low excretion and a little more in the group with high excretion.

Because extraction of the phytoestrogens from urine can be avoided with the current method, it is suitable for the analyses of a large number of samples in epidemiological studies. However, usually plasma samples are used and for that purpose we have developed highly sensitive plasma methods [30–32]. The recently published plasma enterolactone method [32] has been modified to also include genistein and daidzein using only 200 μl plasma samples for duplicate analyses of all three phytoestrogens (submitted for publication).

It is concluded that the method developed for the three main urinary phytoestrogens fulfills the reliability criteria with the exception of the overestimation of genistein and slight overestimation of enterolactone, probably due to interference with structurally similar metabolites. This is of no disadvantage in routine use because of the good correlation between the reference GC–MS method and the immunoassay and as long as adequate reference values have been established. The conversion of the TR-FIA values to "GC–MS values" is also possible, if required.

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