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Quantitation of soy-derived phytoestrogens in human breast tissue and biological fluids by high-performance liquid chromatography

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Abstract

A new and reliable HPLC method for the quantitation of daidzein, equol, and genistein in human breast tissue has been developed. The method was applied to biopsies from women undergoing breast reductions, who, prior to surgery, had ingested either a soy isoflavone preparation or a placebo tablet. The results were compared with data collected for urine and serum of the same subjects using standard methods. The limits of detection in the breast tissue homogenate were 24.7 nmol/l for daidzein, 148.0 nmol/l for equol, and 28.4 nmol/l for genistein (S/N of 3). The chromatographic limits of quantitation were 62.5 nmol/l for daidzein and genistein, and 125.0 nmol/l for equol, for which the accuracies were 86.0%, 83.6%, and 81.8%, respectively. The coefficients of variation of these measurements were all below 20% (11.1% for daidzein, 16.4% for genistein, and 13.2% for equol). The sample preparation comprised a concentration step and the absolute limits of quantitation were, therefore, 4.7 nmol/l, 18.8 nmol/l, and 0.94 nmol/l for daidzein and genistein, and 9.4 nmol/l, 37.5 nmol/l, and 1.9 nmol/l for equol in urine, serum, and breast tissue homogenate, respectively. Recoveries were between 70% (±5.6%) in breast tissue homogenate and 100% (±14.1%) in urine and serum for all three compounds. Equol (less than 1 μmol/l homogenate) was found to be the predominant phytoestrogen in breast tissue and its concentrations exceeded those in serum. The concentrations of phytoestrogens were at least 100-fold higher in urine than in serum and breast tissue.

Keywords: Soy; Phytoestrogens; Daidzein; Equol; Genistein

1. Introduction

Phytoestrogens, particularly soy isoflavones, are known to exhibit various health-beneficial effects [1–7] including relief of menopausal symptoms and preventive effects in the development of cardiovascular diseases and hormone-dependent cancers. Isoflavones such as genistein and daidzein, and equol, a daidzein metabolite formed by the gut microflora after ingestion [3,5], demonstrate oestrogenic responses when administered to humans and

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animals, but the oestrogenic activities are several hundred-folds weaker than the oestrogenicity of 17β-oestradiol [8,9]. However, some foods contain comparatively large amounts of phytoestrogens, including soy and soy products, and plasma concentrations may, therefore, exceed levels of endogenous oestrogens by several orders of magnitude [10].

A number of in vitro studies have shown that isoflavones exhibit biphasic effects on the growth of mammary carcinoma (MCF-7) cells, stimulating proliferation at low concentrations but inhibiting it at high concentrations. A dose-dependent stimulation of MCF-7 breast tumour growth after genistein and soy protein isolate administration in feed has been observed in ovariectomised athymic mice [11]. It was suggested that the low endogenous oestrogen levels resulting from ovariectomies of the animals could afford genistein to be a significant source of oestrogenicity and, hence, stimulate tumour growth. Such effects may also be observed in postmenopausal women with existing oestrogen-dependent breast cancer having low endogenous oestrogen concentrations, but with a consumption of isoflavones through various foods or supplements sufficient to reach plasma levels capable of enhancing the oestrogen-dependent tumour.

These issues raise the need to quantitate phytoestrogen concentrations in human tissues, especially in the breast, as tissue levels of phytoestrogens are likely to determine subsequent biological activity. Several analytical techniques, including high-performance liquid chromatography (HPLC) [12–17], gas chromatography [18–20], capillary electrophoresis [21], time-resolved fluoroimmunoassay [22], enzyme-linked immunoassay [23], and radioimmunoassay [24] have been applied to the quantitation of phytoestrogens in human biological fluids such as urine and serum or plasma [16,25–30], prostatic fluid [30], and human milk [25,31]. However, to the best of our knowledge, quantification of phytoestrogens in human breast tissue has not been reported yet. The widespread use of HPLC with UV detection, the relatively easy sample preparation and potential extension to high-throughput analyses, and the option to quantify simultaneously different sample constituents prompted us to develop a HPLC method to analyse soy-derived phytoestrogens in human breast tissue. In practice, samples of breast tissue were collected from women taking either a soy-based isoflavone preparation or a placebo tablet. Moreover, determination of phytoestrogen concentrations in urine and serum of the same subjects allows further insights into the distribution of phytoestrogens after ingestion.

2. Experimental

2.1. Chemicals and reagents

Daidzein, equol, and genistein were obtained from Extrasynthèse (Genay, France). 4-Hydroxybenzophenone (4-HBPH, internal standard) was from Sigma–Aldrich (Bornem, Belgium). The purities of all analytes were >98%, as assayed by HPLC and ^{1}H NMR (equol and 4-HBPH in $CDCl_3$, genistein and daidzein in DMSO-d6; Varian Mercury 300 MHz NMR spectrometer from Varian, Palo Alto, CA, USA). All solvents were purchased from Biosolve (Valkenswaard, The Netherlands). Tris buffer (0.1 *M*) was made using Tris powder (ICN Biomedicals, Eschwege, Germany) and 0.1 *M* sodium acetate buffer was prepared using sodium acetate from Sigma–Aldrich. β-Glucuronidase/arylsulphatase (98,800 units/ml β-glucuronidase and 1000–5000 units/ml arylsulphatase from *Helix pomatia*), crude lipase (from porcine pancreas), and protease (from *Streptomyces griseus*, 4.4 units/mg solid) were obtained from Sigma–Aldrich. C_{18} solid-phase extraction (SPE) cartridges 6CC (500 mg) were bought from Varian (Middelburg, The Netherlands). HPLC vial 0.1-ml micro-inserts were from Alltech (Lokeren, Belgium).

2.2. Subjects

The subjects were recruited upon contacting the Department of Aesthetic and Reconstructive Surgery at the Ghent University Hospital for the purpose of breast reductions and data from three healthy women aged 30, 45, and 47 years, respectively, are presented here. Brief diet histories were noted and the use of antibiotics in the months prior to surgery, which could interfere with equol production, served as an exclusion criterion. Participants in the study ingested either a soy-based isoflavone preparation (EB605,

containing 100 mg genistein/genistin, 37 mg daidzein/daidzin, and 15 mg glycitein/glycitin, Eko-Bio, Eede, The Netherlands) or a placebo tablet for 5 consecutive days prior to surgery. The study was given ethical approval by the Ethics Committee of the Ghent University Hospital and a written consent form was obtained from each subject.

2.3. Sample collection and preparation

On the day of surgery, samples of urine and blood were collected, along with a breast tissue biopsy. The breast tissue and urine samples were placed in sterile containers, while blood was drawn into sterile Venoject II tubes (4 ml, Autosep®, Terumo Europe, Leuven, Belgium). Breast tissue was immersed in Tris buffer (pH 7.4) after collection (0.222 g of tissue per ml). The breast tissue samples were homogenised using a homogeniser (Kinematica, Luzern, Switzerland) for 10 min. The tissue homogenate (10 ml) was either stored at −70 °C or processed immediately. An extra 5 ml Tris buffer (pH 7.4), 100 mg lipase, and 16 mg protease were added and mixed well, followed by incubation for 1 h at 37 °C. Then, 5 ml sodium acetate buffer (pH 5.0) and 60 μl β-glucuronidase/arylsulphatase were added to the sample, followed by incubation for 6 h at 37 °C. Subsequently, 15 ml methanol including 90 μl internal standard (8 mg 4-HBPH in 100 ml MeOH) and 15 ml hexane were added. The samples were mixed well prior to centrifugation (Eppendorf centrifuge 5810R, Merck Eurolab, Overijse, Belgium) at 3000 *g* for 30 min at 4 °C. The hexane was discarded and the methanol-buffer layer was diluted with 35 ml water.

The solid-phase extraction columns were pre-conditioned with 4 ml ethyl acetate, 4 ml methanol, and 4 ml water, consecutively. After sample application, the cartridges were rinsed with 10% methanol (v/v) in water and the compounds of interest were eluted with 4 ml methanol on a VacMaster 20 sample processing unit (IST, Mid Glamorgan, UK). The solvent was evaporated under a gentle stream of nitrogen and the residue was redissolved in 150 μl 0.05% formic acid in water–methanol 50/50 (v/v).

Whole blood was centrifuged using an Ecco-Praxa 2 (Vel, Leuven, Belgium) at 3000 *g* for 10 min upon collection and serum was frozen at −20 °C prior to analysis, as were urine samples. Urine (2 ml) and serum (0.5 ml) were added to 7 ml sodium acetate buffer (pH 5.0) and 30 μl β-glucuronidase/arylsulphatase. The samples were vortex-mixed and incubated for 3 h at 37 °C, followed by extraction with 90 μl internal standard (8 mg 4-HBPH in 100 ml ethyl acetate) and 7 ml ethyl acetate. The solvent was removed under a gentle stream of nitrogen and the sample was redissolved in 150 μl 0.05% formic acid in water–methanol 50/50 (v/v) prior to analysis.

2.4. Apparatus and chromatographic conditions

All samples were analysed using a Waters 2690 Separations HPLC module equipped with a 996 photodiode array detector (Waters, Brussels, Belgium). Chromatographic processing was done using the Waters Millennium software version 3.2. The reversed-phase silica column was an XTerra™ MS C_{18} 5 μm, 4.6×250 mm (Waters). Analyses were carried out under isocratic conditions with an eluent mixture consisting of 40% solvent B (acetonitrile–methanol 20/80 (v/v)) in solvent A (0.05% formic acid in water, v/v). The flow-rate was 1.5 ml/min, the column temperature was 40 °C, and the run time was set to 20 min, sufficient for all compounds to elute. The injection volume was 30 μl and all samples were analysed in triplicate. Ultraviolet detection was done at a wavelength corresponding to the most intense absorption maximum of each analyte, i.e. 249 nm for daidzein, 230 nm for equol, and 261 nm for genistein. The internal standard (4-HBPH) was detected simultaneously with and at the same wavelength as described above for each compound.

2.5. Calibration curves, recoveries, repeatability, limits of detection and quantitation, and selectivity

Stock solutions in the HPLC mobile phase were serially diluted to obtain a set of calibration standards covering the expected concentration range in the different matrices (0.125–100.0 μmol/l). Each calibration standard was assayed in triplicate and the standard curves were fitted by least squares linear regression. The peak areas versus the nominal concentrations were used to generate the calibration curves and the concentrations of the analytes in

breast tissue homogenate, urine, and serum were determined from these curves. For the determination of recoveries, daidzein, equol, and genistein at 1, 10 and 100 μmol/l in the HPLC eluent ($n=5$) were spiked into breast tissue homogenate, urine, and serum of a subject, in whom these compounds were not detectable.

Both the intra- and inter-assay variations in measurements were determined on breast tissue homogenate, urine, and serum samples containing daidzein, equol, and genistein ($n=5$). The level of acceptance for precision was set at 20%. The limit of detection (LOD) was defined as the analyte concentration that gave a signal-to-noise (S/N) ratio of 3. The limit of quantitation (LOQ) referred to the lowest concentration of analyte ($n=5$) that could be determined with 20% accuracy and precision. The selectivity of the method was studied by analysing several breast tissue homogenate, urine, and serum samples and confirming that there were no interfering substances at the retention times of the compounds of interest under the conditions described.

3. Results and discussion

3.1. Sample preparation

No method to quantify phytoestrogens in human breast tissue has been described in the literature until now. The procedure reported here for quantifying the soy-derived phytoestrogens daidzein, equol, and genistein in human breast tissue using HPLC with diode array detection (DAD) represents a first entry into this analytical field. As we aimed to establish human breast tissue concentrations of daidzein, equol, and genistein simultaneously, in addition to relating them to the urine and serum concentrations in the same subjects, HPLC detection at an appropriate wavelength for each compound proved to be well suited for this purpose. Thus, daidzein was assayed at 249 nm, equol at 230 nm, and genistein at 261 nm.

Samples were obtained from healthy women undergoing aesthetic breast reductions. The composition of the tissues varied greatly, as observed by macroscopic and microscopic evaluation (results not shown). Apart from varying factors such as age, time of sampling during the cycle, and use of exogenous hormones, inter-individual variations cannot be controlled for, while, also, it is not known whether phytoestrogens are localised mainly in epithelial or glandular tissue or in the adipose tissue surrounding these structures. Consequently, we opted for homogenising the human breast tissue samples.

While enzymatic or acid hydrolysis followed by solvent extraction is usually adequate for sample preparation of urine and serum or other biological fluids, human breast tissue is more intricate, mainly because it contains substantial amounts of fat. Moreover, phytoestrogens in the tissue samples could be occupying oestrogen receptor sites or accumulate in the lipophilic tissue due to partitioning from the blood, as has been observed in mammary tissue from rats [12]. Therefore, an extra de-fattening treatment with lipase and solid-phase extraction as well as a tissue-degrading step using protease was found necessary.

The sample preparation of urine and serum presented here has the advantage over existing methods that it is identical for the two matrices and requires fewer preparation steps. The samples were treated with β-glucuronidase/arylsulphatase in order to allow measurement of the total phytoestrogen content in the samples, which is in accordance with the literature [16,17,26,31,32]. However, while other methods frequently include a solid-phase extraction step for urine [16,17,31] or sequential organic acid extractions for both matrices [25,33,34], an acceptable extraction recovery was found by direct extraction of the hydrolysed phytoestrogens.

Daidzein, equol, and genistein in breast tissue, urine, and serum samples were identified by comparison of the retention times with the respective standards (HPLC, UV spectra) (Fig. 1) and by spiking with authentic compounds. Fig. 1 also shows a HPLC chromatogram (UV detection) of a breast tissue sample from a subject on a triple dose of the soy-derived isoflavone preparation, demonstrating adequate separation of daidzein (t_R 7.8 min), equol (t_R 11.0 min), genistein (t_R 13.0 min), and the internal standard, 4-hydroxybenzophenone (4-HBPH; t_R 15.1 min). The calibration curves were linear over the concentration ranges expected to be found in breast tissue homogenate, urine, and serum samples in our study (0.125–100 μmol/l). The

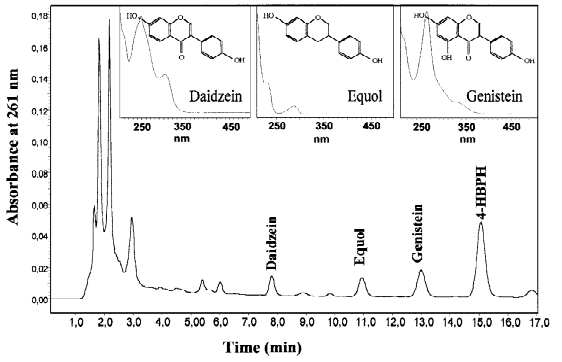

Fig. 1. High-performance liquid chromatogram and ultraviolet spectra of daidzein, equol, and genistein obtained from human breast tissue homogenate of a subject ingesting a triple dose of a soy isoflavone tablet (EB605). The internal standard was 4-hydroxybenzophenone (4-HBPH). For the sake of clarity, only the chromatogram detected at 261 nm is shown.

regression equations were $y = 4.1818x + 1.6228$ ($r^2 = 0.9996$) for daidzein, $y = 1.1981x - 0.0236$ for equol ($r^2 = 0.9998$), and $y = 5.7824x - 1.3667$ ($r^2 = 1.0$) for genistein ($n = 3$ for all concentrations).

3.2. Method characteristics

The intra- and inter-assay repeatabilities of breast tissue homogenate, urine, and serum samples containing daidzein, equol and genistein, were between 0.1 and 16.7% for daidzein, 1.4 and 19.0% for equol, and 0.4 and 9.3% in the three matrices, hence well within a 20% cut-off value. The limits of detection and quantitation were established by injecting standard solutions of different dilutions. The limits of detection were 24.7 nmol/l for daidzein, 148.0 nmol/l for equol, and 28.4 nmol/l for genistein (S/N of 3). The limit of quantitation was 62.5 nmol/l for daidzein and genistein, and 125.0 nmol/l for equol, for which the accuracies were 86.0%, 83.6%, and 81.8%, respectively. Furthermore, the coefficients of variation of these measurements were all below 20% (11.1% for daidzein, 13.2% for equol, and 16.4% for genistein). As samples were concentrated during pretreatment, the absolute limits of quantitation were 0.9 nmol/l for daidzein and genistein, and 1.9 nmol/l for equol in breast tissue homogenate (concentration factor of 66.7), 4.7 nmol/l for daidzein and genistein, and 9.4 nmol/l for equol in urine (concentration factor of 13.3), and 18.9 nmol/l for daidzein and genistein, and 37.5 nmol/l for equol in serum (concentration factor of 3.3).

Recoveries, after spiking with daidzein, equol, and genistein in concentrations of 1, 10 and 100 μmol/l ($n = 5$ for all measurements) were found to be 70% (±5.6%) in breast tissue homogenate and 100% (±14.1%) in urine and serum. The recoveries did not appear to be dependent upon the concentration of the analytes, while the recoveries of the breast tissue homogenate samples might be increased with repetitive extractions (not pursued in the present work). Although exhaustive de-fattening was undertaken using lipase and solid-phase extraction, it is feasible that interferences from the complex tissue matrix lowered extraction efficiencies. A mixture of methanol and buffer, in addition to an extraction step with hexane, resulted in selective extraction of the analytes of interest (Fig. 1). Ethyl acetate was used for highly efficient extraction of daidzein, equol, and genistein from urine and serum, as was found previously [25,31].

As our goal was to investigate the total phytoestrogen content in various samples rather than to distinguish between glycosides and aglycones, enzymatic hydrolysis with β-glucuronidase/arylsulphatase was carried out prior to extraction. Although the enzymes and conditions for hydrolysis of urine and serum samples are largely similar in most studies, the incubation times vary greatly, from 0.5 h to overnight incubations [14,22,25,33–41]. In our hands, a hydrolysis time of 6 h at 37 °C was considered to be optimal for releasing the isoflavones from the respective conjugates in the breast tissue samples (data not shown). Comparison of the data before and after enzymatic hydrolysis suggests that the phytoestrogens in the human breast occur mainly as glucuronide and/or sulphate conjugates. A similar observation has been made for human milk [31].

3.3. Method application

To highlight the quantitation of soy-derived phytoestrogens in breast tissue, data from one subject on the soy-derived isoflavone preparation and one subject on the placebo tablet, in addition to one subject ingesting a triple dose, are presented (Figs. 2 and 3). Fig. 2 shows the concentrations of daidzein, equol, and genistein in breast tissue homogenate,

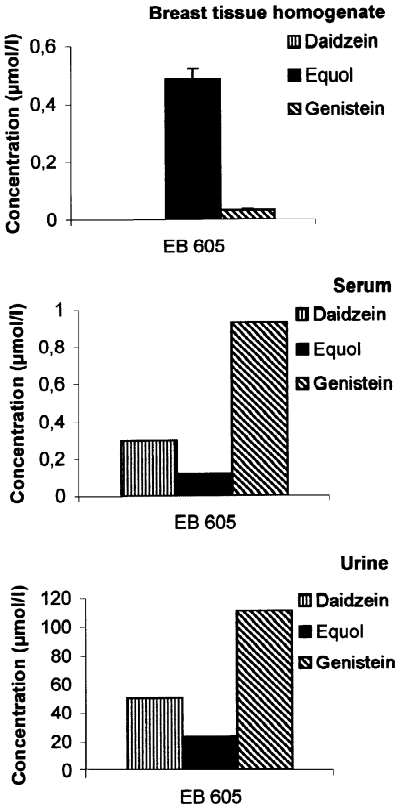

Fig. 2. Daidzein, equol, and genistein concentrations in breast tissue homogenate, serum, and urine in a subject ingesting a soy isoflavone tablet (EB605). The levels of these in breast tissue homogenate and serum in the subject ingesting a placebo tablet were below the limit of detection, while the levels found in urine were very low, 0.32 μmol/l for genistein and 0.15 μmol/l for equol. The results are expressed as μmol/l±SD. All samples were analyzed in triplicate. The concentrations of genistein and equol in the breast tissue homogenate in the subject ingesting the isoflavone tablet correspond to 4.16 μg/g and 52.98 μg/g breast tissue, respectively.

urine, and serum, respectively, of one subject on the soy-derived isoflavone preparation versus one subject on the placebo tablet. A most remarkable observation is the large predominance of equol (at least 10-fold with respect to the concentrations of daidzein and equol) in the breast of the subject ingesting a soy-derived isoflavone preparation. The equol concentration was even higher in breast tissue than in serum.

The results for urine and serum are entirely different, since genistein outweighed in both bio-

Fig. 3. Daidzein, equol, and genistein concentrations in breast tissue homogenate in a subject ingesting a single dose of the soy isoflavone tablet (EB605) compared with a subject ingesting a triple dose of the same tablet. The results are expressed as μmol/l±SD. All samples were analyzed in triplicate. The concentrations of daidzein, equol, and genistein in the subject ingesting a single dose of the isoflavone tablet are described in Fig. 2, while those in the subject ingesting a triple dose of the tablet correspond to 35.1, 681.7, and 16.0 μg/g, breast tissue, respectively.

logical fluids the concentrations of daidzein and equol, as was also found by others [42]. Interestingly, the patterns in urine and serum are similar. Taking into account that the soy-derived isoflavone preparation contained 100 mg genistin/genistein and 37 mg daidzin/daidzein, it can be concluded that the concentrations of both isoflavones in urine and serum are comparable. Concentrations of the three compounds in urine were at least 100-fold higher than in breast tissue homogenate and serum (all concentrations expressed in μmol/l for comparison purposes). The concentrations of daidzein, equol, and genistein were very low or beyond the detection limit for breast tissue homogenate, urine, and serum in the subject ingesting the placebo. In order to quantify these levels or establish breast tissue homogenate concentrations in subjects ingesting lower dosages of phytoestrogens, a more sensitive detection system could be applied such as HPLC with fluorescence or mass spectrometric detection. Furthermore, it should be noted that, as the extraction efficiency in breast tissue homogenate was 70%, the concentrations are slightly underestimated. Evidently, a larger number of subjects are needed in order to establish average concentrations of soy-derived phytoestrogens in breast tissue, urine, and serum, as literature data show great inter-individual variations in all biological matrices studied [40,43–45].

Confirmation of the reliability of the method was

found in the increased concentrations of the compounds of interest in a subject ingesting a triple dose of the soy-derived isoflavone preparation (Fig. 3). The equol concentration of 6 μmol/l is noteworthy. An increase in phytoestrogen concentrations in breast tissue with increasing intake may have important implications with respect to reaching optimum concentrations at a specific tissue site. However, it is possible that tissue concentrations of these compounds will show marked qualitative and quantitative differences depending on type of soy food or supplement ingested [42].

4. Conclusions

A new and reliable HPLC method with UV detection was developed for the quantitative determination of soy-derived phytoestrogens, daidzein, equol, and genistein, in human breast tissue. The procedure was highlighted using a restricted number of breast tissue samples obtained in a real clinical situation. At least 10-fold higher concentrations of equol, albeit less than 1 μmol/l homogenate, were found in comparison to those of daidzein and genistein, suggesting that equol may be quantitatively the main phytoestrogen in the breast following soy intake. The concentration of equol was even higher in the breast than in serum, while the phytoestrogens were predominant in urine (low μmolar ranges). The concentrations of daidzein, equol, and genistein increased according to the triple dose ingested by one subject. Applications to more subjects are ongoing and the breast tissue samples will be both biochemically and histologically examined to account for the heterogeneity of the samples.

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