Phytoestrogen Concentrations in Serum from Japanese Men and Women over Forty Years of Age

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ABSTRACT Asian individuals have much lower incidences of prostate and breast cancer than populations from Western developed countries. They also consume a lower fat, higher fiber diet, with a large intake of phytoestrogens. These phytoestrogens may protect against hormone-dependent cancers and other diseases. Our study used established gas chromatography-mass spectrometry (GC-MS) methodologies to measure the concentrations of four phytoestrogens (daidzein, genistein, equol and enterolactone) in serum samples obtained from Japanese men (n = 102) and women (n = 125) > 40 y old. The results were compared with those obtained with samples from the UK. The Japanese men and women had higher (P < 0.001) concentrations of circulating daidzein, genistein and equol than individuals from the UK. The mean concentration of genistein in Japanese men, for example, was 492.7 nmol/L, compared with 33.2 nmol/L in men from the UK. The two populations, however, had similar serum concentrations of enterolactone. Furthermore, 58% of the Japanese men and 38% of the Japanese women had equol concentrations > 20 nmol/L, compared with none of the UK men and 2.2% of the UK women. These results support previously published GC-MS results from studies with low numbers of samples. J. Nutr. 132: 3168–3171, 2002.

KEY WORDS: • phytoestrogens • soy • Japanese • serum

Recent epidemiologic studies have highlighted the differences between countries in the incidence of many diseases, including prostate, breast, endometrial and ovarian cancers, and coronary heart disease (1,2). Asian men and women, from China or Japan, for example, have much lower incidences of prostate and breast cancer than populations from the Western developed countries (3). When Chinese and Japanese people migrate to Hawaii or to mainland North America, however, their incidences of both prostate and breast cancer rapidly increase to approximately half that of the indigenous population (4,5), suggesting that dietary and environmental factors, rather than racial characteristics, are involved. Vegetarian men in the United States also have a lower incidence of prostate cancer than their omnivorous counterparts (6).

Both Asian and vegetarian populations consume a low fat, high fiber diet, and also have a large intake of plant-derived estrogenic compounds known as phytoestrogens. The role of these compounds as protective agents against hormone-dependent cancers and coronary heart disease has recently attracted increasing interest (7,8). Others, however, have highlighted the possibility that dietary phytoestrogens are harmful, especially in the developing fetus (9,10).

The two principal classes of phytoestrogens are the isoflavonoids and mammalian lignans (11), although many flavonoids have estrogenic properties (12). Generally, the presence of isoflavonoids in plants is limited to legumes, and the richest source is soybean (13–15). Soybeans contain glycoside conjugates of the isoflavonoids genistein and daidzein, which can be metabolized by gut bacteria to produce their respective aglycones. These can then be further metabolized to the nonestrogenic p-ethylphenol and the estrogenic isoflavon, equol, respectively. The richest sources of plant lignans are linseed (flaxseed) and other oilseeds such as sesame (16), which contain secoisolariciresinol and matairesinol, respectively. After ingestion, these are metabolized by colonic bacterial flora to the weakly estrogenic enterolactone and enterodiol. These isoflavonoid and lignan metabolites can then be absorbed from the gut and appear in blood and urine as glucuronide and sulfate conjugates (11).

The concentrations of isoflavonoids are high in the urine (17) and plasma (18–21) of Japanese men and women whose traditional foodstuffs contain large amounts of soybeans in the form of bean curd (tofu), soybean milk, miso and tempeh. The concentrations of lignans, on the other hand, are high in the urine of vegetarians (22) whose diet contains whole grain cereals, vegetables and fruits.

In this study we describe the use of a robust gas chromatography-mass spectrometry (GC-MS)2 method for the determination of the concentrations of the isoflavonoids, genistein, daidzein and equol, and the lignan, enterolactone, in serum samples obtained from a large group of Japanese men and women > 40 y old. Although dietary records were not taken, these individuals were more likely than younger men and women to be consuming a traditional Japanese diet. The phytoestrogen concentrations obtained were compared with those in serum samples from the UK population (23,24) and with those obtained in the other limited studies mentioned above (18–21).

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2 Abbreviations used: BSTFA, N,O-bis(trimethylsilyl)trifluoroacetamide; CI, confidence interval; GC-MS, gas chromatography-mass spectrometry; TR-FIA, time resolved fluoroimmunoassay.
SUBJECTS AND METHODS

Chemicals and reagents. All solvents were purchased from Fisher Scientific UK (Leicestershire, UK) and were fractionally distilled before use. The β-glucuronidase/aryl sulfatase preparation (Hexapomata) and dichlorodimethylsilane were obtained from Sigma-Aldrich (Dorset, UK). Sephadex LH-20 was obtained from Pharmacia Biotech UK (Buckinghamshire, UK) and N,O-bis(trimethylsilyl)triﬂuoroacetamide (BSTFA) from Pierce & Warner (Chester, UK). Deuterated internal standards were a gift from Professor Herman Adlcreutz, Folkhalsan Research Center, Helsinki, Finland. Daidzein and genistein were obtained from Toronto Research Chemicals, Toronto, Canada; enterolectone was a gift from Dr. A. W. Sim, Organon UK, Newhouse, Lanarkshire, Scotland, and equol was a gift from Dr. Nigel Botting, University of St. Andrews, Scotland.

Serum samples. Serum samples were obtained from 102 Japanese men between the ages of 40 and 85 years (mean 64.4) and 125 Japanese women between the ages of 40 and 89 years (mean 67 y) who were not fasting. All subjects were considered to be healthy, and were not taking medication or attending hospital outpatient clinics. Blood samples were taken at the time of routine health examinations in Tokyo to test for hyperlipidemia, diabetes, or abnormal liver function. All samples were collected in the autumn between 0900 and 1200 h and were from individuals who did not show any signs of the above conditions. Recent dietary histories were not collected. Samples were shipped to Cardiff on dry ice and stored at −80°C until phytoestrogen analysis. Samples from the UK population had been collected and analyzed for phytoestrogens for the purposes of other studies (23,24).

Sample preparation. Serum samples were allowed to thaw to room temperature and an aliquot (300 μL) dispensed, by weight, into a silanized B19 test tube. Sodium acetate buffer (0.1 mol/L, pH 5.0, 1.0 mL), an internal standard cocktail in methanol containing d4-genistein, d4-daidzein, d4-equol and d6-enterolactone (50 μL), and β-glucuronidase/aryl sulfatase enzyme (1000 U/sample) were added. Each sample was adjusted to 2.5 mL with sodium acetate buffer and the hydrolysis of conjugotes allowed to proceed overnight at 37°C.

The aglycones were then extracted into ethyl acetate (2 mL) and the solvent evaporated under nitrogen at 55°C until phytoestrogens were then eluted with methanol (4 mL). The methanol was removed under nitrogen at 60°C and the dry extract derivatised for GC-MS by forming trimethylsilyl derivatives by reaction with BSTFA at room temperature overnight.

Instrumental analysis. GC-MS was performed using a MD800 bench-top quadrupole mass spectrometer coupled to a GC8000 gas chromatograph [Fisons Instruments (now Thermo), Manchester, UK]. The GC8000 housed a 12 m × 0.32 mm SE54 silica capillary column (1.0-μm phase thickness, Alltech, Lancashire, UK), which was programmed from 90°C to 245°C at 4.9°C/min under a helium pressure of 25 kPa. Calibration standards and sample extracts, both in BSTFA, were injected into a splitless injector which was maintained at 230°C. The interface and source were operated at 250 and 230°C, respectively.

Isotope dilution GC-MS was carried out in the selected ion recording mode. In this technique, the intensities (peak height) of selected ions (normally the molecular ion, M+) for each analyte (e.g., genistein) and each deuterated internal standard (e.g., δ4-genistein) were monitored, recorded and expressed as a ratio (peak height analyte/peak height deuterated internal standard) for each calibrant and sample extract. A calibration curve of peak height ratio against concentration of analyte is then plotted for the calibration standards and the concentrations of the analyte in the sample extracts are read from this. The detection limit for each of the four assays was 0.1 μg/L (equol, 0.417 nmol/L; enterolactone, 0.336 nmol/L; daidzein, 0.394 nmol/L; and genistein, 0.370 nmol/L); data below these limits were regarded as zero.

Statistical analysis. The mean, median, interquartile range around the median and 95% confidence interval (CI) around the mean were calculated for each group of samples (i.e., samples from men and women from Japan and UK). Student’s unpaired t test was used to compare the means.

RESULTS

The mean concentrations of daidzein, genistein, equol and enterolactone in the serum of Japanese men (n = 102) and women (n = 125) are shown in Table 1. The table also contains the median, s.d., interquartile range around the median, 95% CI around the mean and ranges for each group. For comparison, the concentrations of the same compounds in the serum of UK men (n = 43) and women (n = 133) are also shown.

| Table 1 | Phytoestrogen concentrations in the sera of men and women from Japan and the UK1,2 |
|-----------------|--------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                | UK                | Japan           | UK              | Japan           | UK              | Japan           | UK              | Japan           |
| Equol           | 24.4              | 32.6            | 17.9            | 282.5**         | 33.2            | 492.7***        |                 |                 |
| (95% CI)        | (±7.3)            | (±11.4)         | (±5.6)          | (±7.2)          | (±8.0)          | (±116.6)        |                 |                 |
| Median          | 18.8              | 10.8            | 14.1            | 148.5           | 26.5            | 287.7           |                 |                 |
| (Q2–Q1)         | (19.6)            | (29.4)          | (14.0)          | (312.6)         | (25.2)          | (435.4)         |                 |                 |
| SD              | 24.5              | 58.7            | 18.7            | 374.5           | 26.8            | 600.9           |                 |                 |
| Range           | 0–6.3             | 0–1922          | 1.5–145         | 0–323           | 0.9–99.6        | 2.8–2273        | 0.5–118         | 0–4092          |
| Enterolectone   | 18.7              | 22.7            | 12.5            | 246.8**         | 27.7            | 501.9**         |                 |                 |
| (95% CI)        | (±5.5)            | (±6.8)          | (±1.8)          | (±6.9)          | (±4.8)          | (±125.8)        |                 |                 |
| Median          | 10.8              | 9.7             | 109.5           | 234.0           | 19.2            | 234.0           |                 |                 |
| (Q2–Q1)         | (16.5)            | (26.9)          | (12.3)          | (246.3)         | (18.9)          | (429.9)         |                 |                 |
| SD              | 16.4              | 31.3            | 10.8            | 370.2           | 28.1            | 717.5           |                 |                 |
| Range           | 0.3–102           | 0–129           | 0.8–65          | 0–2407          | 0.4–157         | 0–4192          |                 |                 |

1 For men, n = 102 (Japan) and n = 43 (UK); for women, n = 125 (Japan) and n = 133 (UK).
2 Abbreviations: CI, confidence interval; Q2–Q1, interquartile range.
3 *** P < 0.001 vs. mean concentration in UK samples.
The concentrations of daidzein, genistein and equol were higher (P < 0.001) in serum from Japanese men and women than in samples collected from their UK counterparts. Serum enterolactone concentrations, however, did not differ between the groups. When Japanese men and women were compared, the serum concentrations of the four phytoestrogens did not differ between them. Because not all individuals are capable of metabolizing daidzein to equol (11), it is also interesting to note that 58% of the Japanese men and 38% of the Japanese women had equol concentrations > 20 nmol/L. In comparison, none of the UK men and only 2.2% of the UK women had equol concentrations of > 20 nmol/L.

**DISCUSSION**

The importance of the structural similarity of the isoflavonoids and lignans to mammalian estrogens and possible effects on cancer prevention were first proposed in the early 1980s in publications by Setchell and by Adlercreutz [reviewed in (11)]. Since then, the literature on the possible health benefits of these compounds has expanded exponentially.

The study presented here is the first to measure, by GC-MS, the concentrations of phytoestrogens in the serum of a large number of Japanese men and women. Although dietary questionnaires were not completed, these individuals are more likely than those < 40 y old to consume a traditional Japanese diet containing large amounts of soybeans. The mean concentrations obtained for the isoflavonoids daidzein (282.5 nmol/L) and genistein (492.7 nmol/L) in the serum of Japanese men with a mean age of 64 y were 15.8 and 14.8 times, respectively, the means in samples from men living in the UK. In a previous study, however, and using similar GC-MS methods, Adlercreutz et al. (19) reported mean concentrations of daidzein and genistein in the plasma of 14 healthy 55-y-old Japanese men consuming a traditional diet of 107 and 276 nmol/L, respectively. Although not included in the data just quoted, Adlercreutz et al. (19) pointed out that one subject had a plasma daidzein level in excess of 900 nmol/L and another had a plasma genistein concentration in excess of 2400 nmol/L. The results from our study are therefore generally higher than those observed by Adlercreutz et al. (19), even though the samples in both studies were collected in the morning from subjects who were not fasting.

The mean concentrations of the isoflavones, daidzein and genistein, in the serum of Japanese women with a mean age of 67 y were 19.8 and 18.1 times, respectively, the concentrations obtained in serum samples of women living in the UK. Furthermore, the serum concentrations of daidzein and genistein for Japanese men, 282.5 and 492.7 nmol/L, were very similar to those of Japanese women, 246.8 and 501.9 nmol/L, respectively. These values for Japanese women are higher than the nine samples analyzed by Adlercreutz et al. (18). It should be noted, however, that the subjects included in that paper were young, pregnant women and that the samples were taken at admission when delivery had started and at a time when the women had not eaten for several hours.

A recent study by Uehar et al. (20) measured the plasma concentrations of genistein, daidzein and enterolactone in a large number of Japanese (n = 111) and Finnish (n = 87) women by time resolved fluoromunooassay (TR-FIA). The mean plasma concentrations of daidzein, genistein and enterolactone in the Japanese women were 118, 406.8 and 13.3 nmol/L, respectively. In a similar study (19) of Japanese women (n = 106), again measured by TR-FIA, the mean plasma concentrations of daidzein and genistein were 111.7 and 307.3 nmol/L, respectively. Although the use of TR-FIA for the measurement of these compounds in serum and plasma is still a relatively recent innovation, the authors (20) claim good correlations (r = 0.956 for genistein and r = 0.951 for daidzein) between TR-FIA and GC-MS results. Overall, the mean plasma concentrations of genistein and daidzein in Japanese women in the two studies above (20,21) are still lower than the corresponding values we report here. There are obvious differences in methodology, i.e., GC-MS vs. TR-FIA, but there are other differences, also. In the study of Uehar et al. (20), the plasma samples were taken from younger women (40–60 y old) after an unspecified fasting period, whereas in our study, the serum samples were collected from nonfasting older women (40–89 y old) whose dietary histories were unknown.

Of the Japanese men in our study, 58% had equol concentrations > 20 nmol/L. This percentage is much higher than the 30% obtained in studies in which Western men and women were challenged with soy to determine how many of them were able to metabolize daidzein to equol (11,15,23). Of the women in this study, however, only 38% had equol concentrations > 20 nmol/L. It is possible that equol might play an important role in phytoestrogen action. Although high concentrations of equol were responsible for “clover disease” in sheep in Western Australia (25), the role of lower, but still substantial concentrations, such as those observed in this study, in humans has yet to be established. It is interesting to note, however, that premenopausal women who excrete equol have plasma hormone profiles associated with a lowered risk of breast cancer (26). Furthermore, the study of Ingram et al. (27) showed that women who excreted more equol were at a lower risk of developing breast cancer.

The mean serum concentrations of the lignan, enterolactone, in Japanese men and women (32.6 nmol/L and 22.7 nmol/L, respectively) are very similar to those observed for other Asian populations and also for men and women from the UK (24).

In conclusion, therefore, we have demonstrated that Japanese men and women > 40 y old have high circulating levels of isoflavonoids but low circulating levels of lignans. In addition, a high proportion of the men can convert daidzein to equol. These results support previously published GC-MS results from low numbers of samples. Furthermore, they were obtained using a well established GC-MS method that has long been regarded as the gold standard for the measurement of phytoestrogens. Although newer immunological methods (e.g., TR-FIA) are being developed, they have yet to be fully accepted.

It is possible that the recent increases in the incidence of hormone-dependent cancers in Asian countries such as Japan (28,29) are because of the current trend in these countries of abandoning the traditional soy-rich diet in favor of more Westernized foods. We are currently collecting samples from younger (20–30 y old) Japanese men and women to investigate whether such changes in diet are reflected in the circulating levels of phytoestrogens.

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**LITERATURE CITED**
