Determination of lignans and isoflavonoids in human female plasma following dietary supplementation

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Abstract

Plasma levels of the lignans enterodiol and enterolactone, and also the isoflavonic phyto-oestrogens daidzein, equol and genistein, are reported for postmenopausal Australian women consuming a traditional diet supplemented with linseed, soya flour or clover sprouts. Analysis was performed by gas chromatography-mass spectrometry, after enzymatic hydrolysis and ion-exchange chromatography. Following linseed supplementation, combined levels of enterolactone and enterodiol reached 500 ng/ml, whereas after soya flour or clover sprouts the respective concentrations of equol, daidzein and genistein reached 43, 312 and 148 ng/ml. Not all subjects were able to produce equol from daidzein. The possible relationship and role of these weak dietary oestrogens as restraining factors in the development of hormone-dependent cancers in Asian populations is discussed.

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Introduction

Linseed (flaxseed) contains the plant lignans matairesinol and secoisolariciresinol as diglycosides (Axelson *et al.* 1982); these are metabolized by bacterial enzymes in the gut to enterolactone and enterodiol respectively (Borriello *et al.* 1985). These mammalian lignans were first observed in the urine of the vervet monkey (Setchell *et al.* 1980*a*) and have since been determined, usually by gas chromatography-mass spectrometry (GC-MS), in the urine of women (Setchell *et al.* 1980*b*) and many other human and other animal biological fluids and excreta (for review see Setchell & Adlercreutz 1988).

Soya is a rich source of the glycosides of daidzein and genistein (Setchell *et al.* 1987), in some subjects daidzein may be metabolized to the isoflavan equol (Setchell *et al.* 1984). Similarly, clover sprouts contain the isoflavonoid formononetin which may also be metabolized, via daidzein, to equol (Price & Fenwick 1985). Equol was first identified in the urine of pregnant mares (Marrian & Haslewood 1932), and was shown to be the agent responsible for the infertility syndrome referred to as 'clover disease', which adversely influenced the sheep breeding industry in Western Australia (Shutt 1976). Adlercreutz and his colleagues have subsequently identified the presence of the isoflavan equol in the urine of chimpanzees (Adlercreutz *et al.* 1986*b*) and women (Adlercreutz *et al.* 1982), as well as some isoflavones, including genistein (Adlercreutz *et al.* 1991).

Little information is available concerning the concentrations of isoflavonoids and lignans in human (or other animal) plasma. Levels of free non-conjugated enterolactone in male plasma have been reported to range from 3·1 to 6·3 ng/ml; total levels were much higher between 11 and 38 ng/ml (Dehennin *et al.* 1982). In human semen the values were approximately fivefold greater. From our own laboratories, plasma levels of 1·6 ng/ml and 38 ng/ml for free and total enterolactone have been reported (Finlay *et al.* 1991). Recently Adlercreutz and his colleagues have published data from the Finlandia study giving the concentrations in plasma of three lignans (matairesinol, enterodiol and enterolactone) and four isoflavonoids (daidzein, 0-desmethylangolensin, equol and genistein) for the biologically active free and sulphate fractions and also the inactive glucuronide fraction (Adlercreutz *et al.* 1993*b*).

This communication relates to the determination, by single ion recording GC-MS, of the concentrations of the mammalian lignans, enterolactone and enterodiol, the isoflavones daidzein and genistein and also the isoflavan equol (Fig. 1), in plasma samples from postmenopausal Australian women taking food supplements of linseed, soya and clover sprouts (Wilcox *et al.* 1993).

Materials and Methods

Chemicals and reagents

All solvents were purchased from BDH/Merck (Poole, Dorset, UK) and were fractionally distilled before use. The β-glucuronidase/aryl sulphatase preparation (*Helix pomatia*) was obtained from Sigma Chemical Company (Poole, Dorset, UK). Sephadex LH-20 was supplied by

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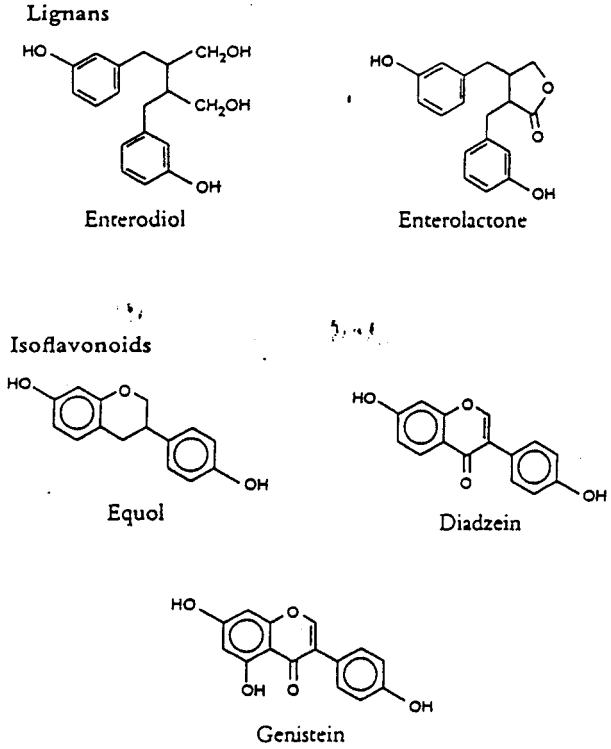

FIGURE 1. Chemical structures of lignans and isoflavonoids.

LKB-Pharmacia (Uppsala, Sweden), dichlorodimethylsilane (DCMS) by Aldrich Chemical Company Ltd (Gillingham, Dorset, UK) and N,0-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) was obtained from Pierce Chemical Company (Rockfield, IL, USA). Test foodstuffs were supplied in Australia by local agents as discussed later.

Standards and internal standards

Equol, daidzein and genistein were purchased from Plantech. UK, Ltd (The University of Reading, Berks, UK). Enterolactone and enterodiol were generously donated by Dr A W Sim, Organon Laboratories, Newhouse, Lanarkshire, UK. All deuterium-labelled compounds were gifts from Professor H Adlercreutz (Meilahti Hospital, Helsinki, Finland).

Glassware

All glassware, including evaporation vials and Pasteur pipette columns, was deactivated by silanization in 10% (v/v) DCMS in toluene, followed by washing once with toluene and twice with methanol.

Study design

The investigation was carried out over 10 weeks, comprising a 2-week lead-in period, followed by a 6-week experimental and a subsequent 2-week washout period. A regular background diet was maintained throughout the study. During the experimental period, between weeks 3 and 8, the diet was supplemented with soya, clover sprouts or linseed, each for 2-week periods. Subject groups were allocated as shown in Table 1.

Subjects

Twenty-nine volunteer subjects were recruited. They were at least 12 months postmenopausal, non-smokers, in good general health and not currently being treated with either oestrogens or any other medications known to affect hormonal status directly, nor to have used antibiotics during the previous 3 months. One subject, however, was treated with doxycycline for a respiratory tract infection before the study commenced, and another received erythromycin stearate during the fifth week of the study. Four subjects withdrew in the early part of the study and two withdrew at later intervals; 23 subjects completed the study.

Informed written consent was obtained from all volunteer subjects and the investigation was approved by Monash Medical Centre (Advisory and Ethics) Committee.

TABLE 1. Study design and allocation of subject groups

	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Week 9	Week 10
Group										
A	Baseline		Clover		Linseed		Soya		Washout	
B	Baseline		Clover		Soya		Linseed		Washout	
C	Baseline		Linseed		Clover		Soya		Washout	
D	Baseline		Linseed		Soya		Clover		Washout	
E	Baseline		Soya		Clover		Linseed		Washout	
F	Baseline		Soya		Linseed		Clover		Washout	
Sampling day	7	14	21	28	35	42	49	56	63	70

There were five subjects in group B and four in the rest.

Test foodstuffs

Soya Full fat, steam-treated soya flour was purchased from Soy Products of Australia Pty Ltd, Bayswater, Victoria, and was divided into 14 day rations, each containing 635 g. This provided a daily dose of 45 g plus an extra 5 g to allow for spillage. To facilitate compliance, no specific recommendation was made as to how it should be consumed.

Red clover Red clover seeds, for sprouting, were purchased from Soulfood (Fitzroy, Victoria, Australia) and sent to Beans Pty Ltd (Dandedong, Victoria, Australia) to be sprouted. One weeks supply, equivalent to 10 g dry seeds was given fresh to each subject on days 1 and 8 of the 2-week block. Subjects consumed the sprouts as additions to their daily diets.

Linseed This was purchased from Soulfood, and divided into 14-day rations each of 355 g, thereby providing a daily intake of 25 g together with an extra 5 g to allow for spillage.

Overall, subject compliance with the protocol was good. Five subjects, however, did not consume the full amount of soya; three did not consume all the red clover and two incorrectly measured the daily ration of linseed.

Sample collection

Non-fasting blood samples were taken on day 7 of each 2 weeks throughout the study, and also 6 weeks after the completion of the study. Although a particular time was not specified, subjects generally came in at the same time of day as for the fasting sample. At the end of each 2 weeks, blood samples were also taken from subjects between 0800 and 1230 h after a 12-h fast. Plasma was separated and the samples frozen at −20 °C until transported by carrier to the Tenovus Cancer Research Centre, University of Wales College of Medicine, Cardiff, UK. The samples were shipped in solid CO_2 and arrived in Cardiff still frozen. Samples were further stored at −20 °C until analysed.

Experimental procedures

Preparation of samples for GC-MS Plasma samples were initially incubated with a washed preparation of β-glucuronidase to hydrolyse conjugates. This was followed by solvent extraction and ion-exchange chromatography to isolate a diphenolic fraction which was derivatized for GC-MS.

Washing of enzyme *Helix pomatia* juice (300 µl) was diluted with 0·1 M sodium acetate buffer (pH 5·0; 5 ml) and applied to a 300 × 10 mm prewashed (water jacketed) column of Amberlyte XAD-2 (Rohm and Haas, Philadelphia, PA, USA) at 55 °C in 0·1 M sodium acetate, (pH 5·0). The column was eluted at a slow drop rate (1 ml/min), the coloured fraction collected into a B19 test tube and extracted with 3 vol. (30 ml) of freshly fractionated diethyl ether. Most of the organic layer was removed with a Pasteur pipette and discarded, residual ether was blown away under nitrogen gas at 50 °C in a water bath. After cooling to room temperature, the washed enzyme was stored at −20 °C until use. The Amberlyte XAD-2 column was washed with 3 column volumes each of 0·1 M sodium acetate (pH 5·0), distilled water and finally methanol to prepare for re-use.

Sample processing An aliquot (1 ml) of the plasma sample was dispensed into a silanized B19 test tube; an equal volume of 0·1 M sodium acetate, (pH 5·0), was added, followed by the washed β-glucuronidase (*Helix pomatia*, 1000 Fishman Units) and deuterated internal standard 'cocktail' (50 µl). The cocktail contained tetradeuterated derivatives of equol, daidzein and genistein and also hexadeuterated derivatives of enterodiol and enterolactone, all at a concentration of 100 ng/ml. The sample preparation was thoroughly mixed and then incubated at 37 °C for 16 h. The hydrolysate was extracted with fractionated diethyl ether (15 ml), the organic fraction water-washed (1 × 3 ml) and then dried under a stream of nitrogen at 50 °C. Short (1 × 3 cm) columns of diethylaminohydroxypropyl–Sephadex LH-20 (in the hydroxide form: Setchell *et al.* 1976) were prepared in silanized Pasteur pipettes. The columns were prewashed with methanol (2 × 1 ml) and the hydrolysis extract applied in 2 × 200 µl aliquots of methanol. The columns were washed with methanol (6 × 1 ml) and this eluate discarded, the diphenolic fraction containing the plant oestrogens was collected by washing the columns with methanol saturated with carbon dioxide (6 × 1 ml). The final 4 ml of this eluate was retained and blown dry in a silanized tube at 50 °C under nitrogen. The diphenolic fraction was converted to the trimethylsilyl ether derivatives by reaction with BSTFA. This reagent (20 µl) was added to each dried extract and allowed to stand at room temperature overnight. The derivatized extracts were then stored at −20 °C until analysed by GC-MS. A reagent blank was processed simultaneously with each batch of samples (usually 25). Blank values for each analyte were close to the level of the zero standard and were typically less than 0·01 ng/ml for each analyte. The values quoted in the Tables are uncorrected.

Instrumental analysis GC-MS was carried out using a DANI 3800 gas chromatograph coupled to a VG7070 HS double-focussing mass spectrometer. The fused silica capillary column (30 m × 0·32 mm, 0·25 µ DB-1), operated isothermally at 280 °C, leads directly into the ionization region of the mass spectrometer ion source. Ions were generated by electron impact at 70 eV using a nominal accelerating voltage of 4 kV at an ion source temperature

TABLE 2. Ions monitored and gas chromatography retention times (Rt) for the analyte trimethylsilyl ethers

	Rt (min·s)	Ion monitored[a] (a.m.u.)	m/z[b] (a.m.u.)
Analyte			
Equol	3·26	M^+	386/390
Enterodiol	5·00	M^+–180	410/416
Enterolactone	5·50	M^+	442/448
Daidzein	7·07	M^+	398/402
Genistein	8·00	M^+–72	414/418

[a]M^+ represents the molecular ion of the analyte and M^+–180, M^+–72 the ions produced by a loss of 180 and 72 atomic mass units (a.m.u.) respectively.
[b]m/z represents the mass (m) to charge (z) ratio.

of 250 °C. Helium, purified by passage through an oxygen trap, was used as carrier gas at 1·5 bar. Samples for analysis were injected in BSTFA reagent (1–5 μl) using an all glass falling needle injector. Single ion recording (SIR) was carried out at a resolution of 1000 (10% valley definition) under the control of a VG2035 data system. The ions monitored for each analyte and GC retention times of their trimethylsilyl ether derivatives under these conditions are listed in Table 2. Reference ions to establish SIR programmes were derived from perfluorokerosene introduced independently into the ion source. Data acquisition and computation of peak heights was carried out using standard VG-SIR software in the data system. Peak height ratios were determined for each standard and extract analysed, and were converted to analyte concentrations by reference to a standard curve established for each assay. Calibration data were fitted to a hyperbolic model using an LS1–11/25 microcomputer (DEC, Maynard, MD, USA).

Assay validation

The method employed was based on that described by Setchell *et al.* (1983). As the internal standard is added at the beginning of the procedure the subsequent enzymatic hydrolysis must be complete. To assess this, a plasma pool (20 ml) was prepared containing high concentrations of the four main analytes (7·5, 70, 100 and 11 ng/ml respectively for equol, enterodiol, enterolactone and daidzein) and 1 ml aliquots were incubated in duplicate with 1000, 2000, 3000, 5000 and 10 000 units of washed β-glucuronidase and internal standard as described above. For equol, enterodiol and daidzein, no increase in the analyte/internal standard ratio was observed when the β-glucuronidase concentration was increased from 1000 to 10 000 units. This indicates that for these analytes the hydrolysis is complete with 1000 units of enzyme. However, for enterolactone, a 10% increase in analyte/internal standard ratio was observed when the amount of β-glucuronidase was increased from 1000 to 2000 units, no further increase was observed for 3000, 5000 or 10 000 units. The hydrolysis of enterolactone with 1000 units of β-glucuronidase was only 90% complete and this probably reflects a high concentration of enterolactone sulphate in this sample. However, the concentration of enterolactone in the pool was very high at 100 ng/ml and therefore for most concentrations outside the linseed-supplemented period, the hydrolysis may be considered complete with 1000 units of β-glucuronidase/ml plasma.

Results

To determine the precision of the assays, plasma samples were processed in duplicate. Each value was determined once by GC-MS using a wide-range standard curve (0–20 ng); concentrations above the high standard were obtained by extrapolation of the standard curve. The data from the precision profiles are listed in Table 3: in most cases the coefficients of variation were better than 10% and ranged from 2·5 to 12·9%. These data would seem satisfactory for samples analysed in duplicate over a wide concentration range. All subsequent assays were performed once. Daidzein, enterodiol and enterolactone can be measured with a sensitivity of approximately 0·05 ng/ml, for equol 0·01 ng/ml may be determined.

TABLE 3. Intra-assay imprecision at different concentration ranges for the four analytes

	No. of duplicates	Concentration range (ng/ml)	Median (ng/ml)	% C.V.
Analyte				
Equol	7	0·04–0·06	0·052	10·0
	4	0·06–0·10	0·075	6·7
	7	0·12–2·15	0·30	7·1
Enterodiol	9	0·06–0·30	0·15	12·9
	8	0·30–10·4	0·61	3·3
Enterolactone	11	0·24–1·96	0·79	4·9
	9	2·52–10·53	4·67	12·5
	3	19·90–91·8	22·4	3·2
Daidzein	15	0·23–7·13	2·80	3·9
	7	11·51–83·90	40·6	2·5

TABLE 4. Overall ranges and means for the plasma concentrations of each analyte following dietary supplementation (supplement in parentheses)

	Plasma concentration	
	Range (ng/ml)	Mean (ng/ml)
Analyte		
Equol (soya)	1·28–53·4	31·1
Equol (clover)	9·5–106·3	34·2
Enterodiol (linseed)	1·85–390	106·3
Enterolactone (linseed)	41·8–244	117·5
Daidzein (soya)	2·74–138·4	68·3
Daidzein (clover)	3·51–153·1	49·1

To give an overall view, the ranges of plasma concentrations of the four analytes and mean values obtained for the different dietary supplements are given in Table 4. The values quoted for equol are derived only from those subjects who were able to metabolize daidzein (see later). The data for daidzein and equol are divided into two groups, one for soya and one for clover. Although the former gave a slightly higher mean concentration for daidzein, overall the differences in the amounts of daidzein and equol derived from these two foodstuffs were probably not significant. It must be pointed out that values above the high standard of 20 ng/ml are semi-quantitative and probably give an underestimate.

To date, ten complete sets of plasma samples from the 23 postmenopausal women who completed the study have been analysed by GC-MS. This has provided information on the plasma concentrations of the mammalian lignans enterolactone and enterodiol, and the isoflavonic phytoestrogens equol and daidzein. For two subjects, semi-quantitative data for genistein were also obtained. Not all the data are presented, since marked similarities were observed in the response of the subjects to the various food supplements, so only representative examples are therefore presented.

All subjects so far studied metabolized linseed to produce a marked increase in the plasma levels of enterolactone and enterodiol compared with basal levels, with differences relating only to the magnitude of the response. This is illustrated in Table 5 for subject DW. From a basal plasma concentration of enterodiol of less than 1 ng/ml there was a rapid rise during the linseed-supplementary period, reaching a maximum level of 176 ng/ml, with a rapid fall to baseline values on withdrawal of the supplement. A very similar pattern was observed for plasma enterolactone where a maximum value of 181 ng/ml was obtained. Baseline plasma levels of enterolactone were, however, higher than those of enterodiol by a factor of 5–20. This is a general observation and consistent with the differences in urinary excretion of these lignans previously reported (Adlercreutz *et al.* 1986*a*).

Throughout the experimental period, the subjects consumed their regular diets, although no dietary information was recorded. However some of the participants were probably more 'vegetarian' than others, since a considerable variation in basal non-supplementary lignan concentration was observed. A vegetarian diet, rich in grains, cereals and pulses, has been shown (Adlercreutz *et al.* 1986*a*) to produce a high urinary excretion of enterolactone. This variation is illustrated for subjects DB and LP in Tables 6 and 7 respectively. From these data, it may be seen that the plasma concentration of lignans for subject DB are much higher than those of LP, particularly in relation to enterodiol. These figures may reflect differences in rates of metabolism, with LP metabolizing enterodiol to enterolactone more quickly than DB, or the data may indicate a greater intake of lignan precursors, such as cereals and grains, in the normal diet of subject DB.

No significant increases in plasma lignan levels were observed during the soya- and clover-supplementary periods. However, the concentrations of daidzein and less frequently equol showed a marked increase after consumption of soya and clover sprouts. This is illustrated in

TABLE 5. Plasma concentrations (ng/ml) of lignans and isoflavonoids for subject DW

Week no.	Supplement	Equol	Enterodiol	Enterolactone	Daidzein
1	None	0·53	0·18	3·4	6·24
2	None	0·08	0·34	4·45	3·20
3	Linseed	0·53	176	113	4·46
4	Linseed	0·52	114	181	9·82
5	Soya	0·16	2·3	9·9	72·7
6	Soya	0·30	2·5	5·8	43·8
7	Clover	0·17	0·48	8·1	26·4
8	Clover	0·16	0·62	6·8	40·1
9	None	0·27	0·40	5·4	14·6
10	None	0·21	0·41	5·5	2·6
16	None	0·08	0·51	8·6	3·7

TABLE 6. Plasma concentrations (ng/ml) of lignans and isoflavonoids for subject DB

Week no.	Supplement	Equol	Enterodiol	Enterolactone	Daidzein
1	No sample				
2	None	0·28	2·04	30·5	3·63
3	Clover	33·7	5·15	40·6	43·1
4	Clover	30·3	5·04	45·0	48·3
5	Linseed	7·7	116	97·7	11·5
6	Linseed	3·1	90·6	108·2	3·75
7	Soya	43·5	4·5	28·6	60·6
8	Soya	26·0	1·22	20·5	19·4
9	None	2·2	8·35	77·0	9·06
10	None	1·4	17·8	56·4	5·49
16	None	0·90	2·28	53·9	2·37

TABLE 7. Plasma concentrations (ng/ml) of lignans and isoflavonoids for subject LP

Week no.	Supplement	Equol	Enterodiol	Enterolactone	Daidzein
1	None	0·34	<0·05	5·0	3·9
2	None	0·014	0·26	3·7	1·4
3	Linseed	0·16	49·9	162	2·0
4	Linseed	0·20	6·33	244	1·2
5	Soya	0·14	0·05	8·5	91·4
6	Soya	0·09	0·10	23·1	55·7
7	Clover	0·10	0·10	7·8	82·3
8	Clover	0·08	0·06	6·8	67·3
9	None	0·14	0·21	6·4	4·4
10	None	<0·01	0·24	1·50	5·4
16	None	0·22	0·45	28·2	4·4

Tables 5, 6 and 7 for subjects DW, DB and LP respectively. Thus for subject LP the daidzein concentration rose rapidly to 91·4 ng/ml, remained high throughout the supplementary period, and rapidly returned to baseline levels on withdrawal of the supplement. Both soya and clover sprouts were effective precursors for the formation of daidzein (see Table 4). However, not all subjects were able to metabolize daidzein to equol. Of the 12 subjects studied, only four demonstrated a capacity to produce high plasma levels of equol when challenged with either a soya- or clover-supplemented diet. Thus subject DB (Table 6) was able to metabolize daidzein, producing plasma equol levels in the range 26–43 ng/ml, whereas subjects DW (Table 5), LP (Table 7) and JB (Table 8), did not show plasma equol concentrations elevated over basal levels. This inability of certain subjects to metabolize daidzein to equol is in agreement with previous observations (Setchell *et al.* 1984).

Some data regarding genistein are included for subject JB (Table 8). Soya is confirmed as an effective precursor for this isoflavonoid, with levels up to 148 ng/ml being observed during the supplemented diet. Unpublished data from this laboratory also indicate that clover sprouts are a rich source of genistein. All the data for genistein, however, must be regarded as semi-quantitative since the deuterated internal standard employed is relatively unstable and tends to lose one deuterium during processing (Adlercreutz *et al.* 1991). This subject (JB) also demonstrated the effect of antibiotics on isoflavonoid metabolism. As indicated in Table 8, subject JB took antibiotics during the clover-supplemented period. While some metabolism to daidzein and genistein occurred, the plasma levels of 15 ng/ml were much lower than those observed during the soya period. However, this effect of antibiotics on phyto-oestrogen metabolism was short-lived, much less than the 6-week period previously reported (Adlercreutz *et al.* 1986*a*), as the formation of lignans during the linseed-supplemented period appeared normal 2 weeks later.

Discussion

The data presented here establish the basal plasma levels of the lignans enterolactone and enterodiol, the isoflavones daidzein and genistein and also the isoflavan equol in postmenopausal Australian women consuming their traditional diet. The effects on the plasma levels of these

TABLE 8. Plasma concentrations (ng/ml) of lignans and isoflavonoids for subject JB

	Supplement	Equol	Enterodiol	Enterolactone	Daidzein	Genistein
Week no.						
1	None	0·28	<0·05	3·34	1·4	13·3
2	None	0·20	0·11	4·16	3·6	29·2
3	Soya	0·14	0·10	3·52	64·0	120
4	Soya	0·10	0·23	9·62	75·1	148
5*	Clover	0·07	4·32	3·1	13·3	15·8
6	Clover	0·16	1·49	3·0	14·9	14·3
7	Linseed	0·20	102	99·6	1·3	7·8
8	Linseed	0·20	6·94	59·6	3·7	15·6
9	None	0·18	1·63	3·6	5·6	38·4
10	None	0·07	0·81	3·8	1·9	6·0
16	None	0·10	0·26	3·6	2·4	9·9

*Antibiotics were taken.

compounds of a diet supplemented daily with linseed, soya or clover sprouts is also presented.

In this study, basal concentrations of the lignans in plasma showed a large variability. In most cases, normal dietary values for enterodiol and enterolactone were between 0–5 ng/ml and 0–10 ng/ml respectively, but occasionally levels for enterolactone up to 70 ng/ml were observed. Enterolactone was usually present at 5–20 times the concentration of enterodiol. Maximum combined concentrations of the lignans, during the linseed-supplemented period, approached 500 ng/ml. This value, although representing the total rather than the free fraction, is some 10 000 times the concentration of free oestradiol seen in postmenopausal women (Reed & Murray 1979) and similar to the levels of the anti-oestrogen tamoxifen observed in patients with breast cancer treated with 20 mg/day of this drug (Daniel *et al.* 1981). Urinary excretion of these compounds has been reported to be highest in vegetarians, and the values are lower in patients with breast cancer (Adlercreutz *et al.* 1986*a*, 1982)

Basal plasma levels of the isoflavonoids daidzein and equol were much less variable, probably reflecting a more uniform intake of precursors in the traditional Western diet of these subjects, irrespective of dietary practices. A vegetarian diet is much more likely to influence levels of lignans rather than isoflavonoids. Equol concentrations were generally in the range 0–1 ng/ml, rising to a maximum of approximately 100 ng/ml during the clover- or soya-supplemented periods. Only 33% (four out of twelve) of the subjects were able to metabolize daidzein to equol, a finding which is consistent with other studies (Setchell *et al.* 1984). The significance of this observation remains as yet unknown. Daidzein levels were generally in the ranges 0–10 ng/ml and 20–100 ng/ml for the background and supplemented diets, although a maximum value of greater than 300 ng/ml was observed during this study. Some semi-quantitative data for genistein, with concentrations in plasma up to 180 ng/ml, indicated that both soya and clover sprouts are efficient precursors for this isoflavonoid. It is currently of considerable interest that the consumption of soya products, and hence also the excretion of isoflavonoids, is highest in areas of low incidence of both breast and prostate cancer.

Cancers of the breast and prostate, in their early phase of development, are hormone-dependent and could be influenced by endocrine changes induced by lignans and isoflavonoids. The oestrogenic potency of flavonoids has been well documented by a variety of *in vitro* and *in vivo* assays, and to a lesser extent so has that of lignans. It was the early work of Folman & Pope (1966), which demonstrated that genistein (and coumestrol) could act as anti-oestrogens by markedly inhibiting the utero-vaginotrophic effects of oestradiol, oestrone and diethylstilboestrol. Anti-oestrogenic properties for equol (Tang & Adams 1980) and enterolactone (Waters & Knowler 1982) have also been reported. Some evidence suggests that certain flavonoids inhibit oestradiol binding to the rat type II nuclear receptor (Markaverich *et al.* 1985, 1988) which, in turn, diminishes uterine cell growth. Genistein has also been reported to inhibit the growth of MCF-7 breast cancer cells in culture (Peterson & Barnes 1993). It would seem very reasonable to propose that these weakly oestrogenic compounds act as the 'natural' tamoxifen in Asian and Mediterranean people. Tamoxifen is now being used as intervention or preventative therapy for women at risk of developing breast cancer. These dietary oestrogens may provide a natural form of intervention therapy.

The action of isoflavonoids and lignans may not necessarily involve oestrogen-receptor mechanisms. Genistein is an inhibitor of tyrosine-specific protein kinases (Akiyama *et al.* 1987). These enzymes appear to be necessary for epidermal growth factor function and the action of other growth factors also, which indicates the antiproliferative potential of this isoflavonoid. The lignan, enterolactone, is a moderate inhibitor of aromatase from placenta (Adlercreutz 1990, Adlercreutz *et al.* 1993*a*) and cultured skin fibroblasts (B A J Evans, C Elford, K Griffiths & M S

Morton, unpublished observation) which suggests that the conversion of androstenedione to oestradiol may be inhibited.

Reports (Adlercreutz *et al.* 1987) have shown that these plant compounds may reduce the bioavailability of steroid hormones by stimulation of sex hormone binding globulin synthesis (SHBG) in the liver. Unpublished data (G F Read) from the Tenovus Cancer Research Centre on nine complete sets of samples from this study provide no evidence of an increase in plasma SHBG during the period when the diet was supplemented. An increase in SHBG would result in reduced levels of free plasma hormones. However, throughout the study, plasma levels of oestradiol remained less than 70 pmol/l and were unaffected by the dietary supplement (M L Wahlqvist, H G Burger & G Wilcox, unpublished observation).

In summary, high concentrations of lignans and isoflavonoids are attained in plasma by supplementing the normal diet with small amounts of the linseed, soya or clover products. These compounds may play a significant role in the molecular processes concerned with the pathogenesis of hormone-dependent cancer, with a real possibility that they exercise a restraining influence on the development of hormone-promoted clinical disease.

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