

Original Article

An Improved Method for Determining Serum Isoflavones by HPLC with Coulometric Array Using Bisphenol A as an Internal Standard

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Background : The influence of isoflavones has recently received a great deal of attention because of their potential beneficial and adverse effects. To evaluate the effects of isoflavones on human health, especially for large-scale epidemiological studies, a rapid, economical, and reliable method is needed to determine the levels of isoflavones in human serum.

Methods : We modified Gamache's method for high performance liquid chromatography-coulometric array detection (HPLC-CA) of isoflavones such as by adding bisphenol A as an internal standard to human serum samples. Samples were hydrolyzed with beta-glucuronidase and sulfatase for 3 h at 37°C. After extraction in ethanol, samples were reconstituted in 20% methanol and applied to the HPLC-CA with binary gradient reversed-phase (C18) chromatography. Eight electrodes were set for suitable detection of daidzein, genistein, and equol in serum and complete determination within 30 min. For validation, we measured isoflavone levels in sera from seven volunteers before and after three days of ingesting Soyaflavone E.

Results : Isoflavones were simultaneously measured using 580-mV single voltage detection. Extraction efficiencies were 105.5% for daidzein, 126.6% for genistein, and 81.8% for equol. In addition, the lower limit of detection in serum was 5.0ng/ml for daidzein, 15.0ng/ml for genistein, and 2.5ng/ml for equol.

Conclusions : For detection of isoflavones in human serum, HPLC-CA using bisphenol A as an internal standard is a rapid, sensitive, and reliable method, which can be applied to large-scale human epidemiological studies. Using this method, we were able to identify equol producers in a general Japanese population.

(Key words : isoflavone, HPLC, coulometric array, human serum, equol producer)

Introduction

The influences of isoflavones, especially those derived from soy and soy products, have recently received a great deal of attention due to their possible beneficial effects as well as

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adverse effects⁽¹⁻⁴⁾. Reported beneficial effects include protection against several forms of cancer⁽¹⁻⁵⁾ and reduced risks of cardiovascular disease⁽⁹⁾ and osteoporosis⁽¹⁰⁾ and reported potential adverse effects include thymic and immune abnormalities⁽¹¹⁾. Furthermore, the potential short- and long-term effects on childhood consumption of soy formula are unclear⁽²⁾.

Genistein and daidzein have been identified as the active isoflavones in soy⁽¹²⁾. Equol is a metabolite produced from daidzein by gut microflora⁽¹³⁾. These three chemicals possess estrogenic activity with some biological effects on health, due to their affinity for both the alpha and beta estrogen receptors^(14,15). In addition, these chemicals are known as antioxidants^(16,17).

To evaluate the effects of these chemicals on human health, especially for large-scale epidemiological studies, a rapid, sensitive and accurate method is needed to determine the levels of genistein, daidzein, and equol in human biological samples. In the current studies, we have chosen to use high performance liquid chromatography (HPLC) with coulometric detection array. This system expands the analytical capabilities for multiple component analysis from a single injection and can minimize sample pre-purification steps when dealing with complex matrices such as serum^(18,19). The coulometric array system is based on the use of multiple electrochemical detectors placed in series after the analytical column. This multi-electrode detector is a series of eight electrochemical cells, each set at different potential. As described by Gamache and Franke^(20,21), this oxidizes or reduces the compounds and detects the component chemicals from small differences in their specific oxidation-reduction behaviors.

We present here a modified HPLC-coulometric array method that can rapidly, efficiently, accurately, sensitively, and economically determine daidzein, genistein, and equol levels in human serum. To validate this analytical method, we analyzed the serum from seven healthy volunteers before and after a three-day dosing with isoflavones.

Materials and Methods

Reagents

Daidzein, genistein, glycitein, equol, and β -glucuronidase (from *Helix pomatia*; 100,000units/ml glucuronidase and 5000 units/ml sulfatase activity) were purchased from Sigma Chemical

Table 1. Components of isoflavones in Soyaflavone E

Form of isoflavone	Content (%)
Daidzin	26.77
Genistin	8.01
Glycitin	26.93
Malonyl Daidzin	3.55
Malonyl Genistin	1.14
Malonyl Glycitin	2.98
Acetyl Daidzin	12.20
Acetyl Genistin	4.92
Acetyl Glycitin	11.48
Daidzein	0.72
Genistein	0.14
Glycitein	1.16
Total	100.00

(St. Louis, MO, USA). All other chemicals and solvents used for HPLC were analytical grade reagents and were purchased from Wako Chemical (Osaka, Japan). Soyaflavone E was a generous gift from Fuji Oil (Osaka, Japan). The content of isoflavones in Soyaflavone E was 6.31%, and specific components are listed in Table 1.

High performance liquid chromatography

HPLC analysis was performed using a CoulArray™ 5600 HPLC detection system consisting of two pumps, a high-pressure gradient mixer, a pulse damper, an autoinjector, a thermostatic chamber, a MD-150 column (150 mm long × 4.6 mm inner diameter, C18 Hypersil 3 mm), and a serial array of eight coulometric electrodes, all obtained from ESA Inc. (Chelmsford, MA, USA). Data processing was carried out with CoulArray™ software (ESA Inc.). The mobile phases were 50 mM sodium acetate (pH 4.8) with acetic acid-methanol, 80 : 20 (v/v) (phase A) and 50 mM sodium acetate (pH 4.8) with acetic acid-methanol-acetonitril, 40 : 40 : 20 (v/v/v) (phase B). The gradient was started with 20% mobile phase B initially, which was followed by a linear increase up to 60% over 5 min, 20 min at 60%, a linear increase up to 100% over 5 min, and a final 5 min at 100%. The flow rate was kept constant at 1.0 ml/min, and the eight coulometric electrodes were set at 260, 320, 380, 440, 480, 580, 620, and 680 mV.

Preparation of standards and recovery study

Daidzein, equol, and genistein were each dissolved in DMSO and the individual solutions were stored at -80°C. Working standard mixtures (5000, 3750, 1250, 500, 375, 250, 125, 50, 37, 12.5, 4.4, 2.5 and 0.5 nM) were prepared by dilution of stock standards in 20% methanol/water (v/v). We selected bisphenol A as an internal standard, and added bisphenol A to the serum specimens as well as to the standard solutions at a final concentration of 2.28 mg/ml prior to HPLC analysis. Therefore, a recovery test was performed in triplicate across a wide range of concentrations by spiking serum with isoflavone standards. The levels of standards spiked into the serum for this analysis were 5, 10, 15, 20, 25, and 50 ng/ml for daidzein and glycitein, 10, 15, 20, 25, 50, and 100 ng/ml for genistein, and 2, 5, 5.0, 10, 15, 20, and 25 ng/ml for equol. The recovery rate was calculated after HPLC analysis.

Preparation of human plasma samples

To determine the total isoflavone levels, an 0.2 ml of serum containing 2.28 mg/ml bisphenol A as an internal standard was mixed with β -glucuronidase (50 μ l) and 0.2 ml buffer [0.1 M sodium acetate (pH 5.0) containing 0.1% ascorbic acid (w/v) and 0.01% EDTA (w/v)]. This mixture was incubated for 3 h at 37°C before the addition of 1.2 ml of ethanol. Following centrifugation at 12,000 × g at 4°C for 10 min, 1.0 ml supernatant was transferred to another tube and dried by vacuum centrifugation. Before analysis, the samples were redissolved by sonication for 5 min in 0.1 ml of methanol and 0.3 ml of water. A 50- μ l aliquot of sample solution was used for HPLC analysis.

Sample collection

Blood samples were collected from seven healthy (six men and two women, 27 to 46 years old) who consented to participate in isoflavone ingestion studies. After collecting pre-dosing blood samples, the subjects ingested 5 g of Soyaflavone E powder with 100 ml water around 10 a. m. for three consecutive days. They were asked to keep their ordinary diet without any dietary restriction during this period. On the third day, their blood was collected 6 h after the last ingestion. The sera were stored at -80°C until use in experiments.

Results

Standard mixture and typical chromatogram

In this experiment, eight electrodes in series were used with the electrode voltages set to 260, 320, 380, 440, 480, 580, 620 and 680 mV, which allowed simultaneous determination of the three isoflavones: daidzein, genistein, glycitein, and equol. Figure 1 shows chromatogram from an injection of a mixture containing standard isoflavones (714.9 ng/ml of daidzein, 759.9 ng/ml of genistein, 799.5 ng/ml of glycitein, and 680.6 ng/ml of equol) and an internal standard (2.28 $\mu\text{g}/\text{ml}$ of bisphenol A). The chemicals reacted with the 490, 58, 620, and 680 mV electrode voltages, and distinct signals appeared on channels 5, 6, 7, and 8.

To identify each isoflavone, the data analysis software defines the three distinct signals and a peak cluster. The channel that demonstrates the highest signal within a cluster is denoted as the dominant channel, the channel of the preceding peak is referred to as the leading channel, and the channel of the subsequent peak is called the following channel. In this analysis, the three isoflavones demonstrated the largest peak areas at 580 mV; bisphenol A, in particular, showed a strong and sharp signal pattern. Thus, we assigned 580 mV as the dominant channel (channel

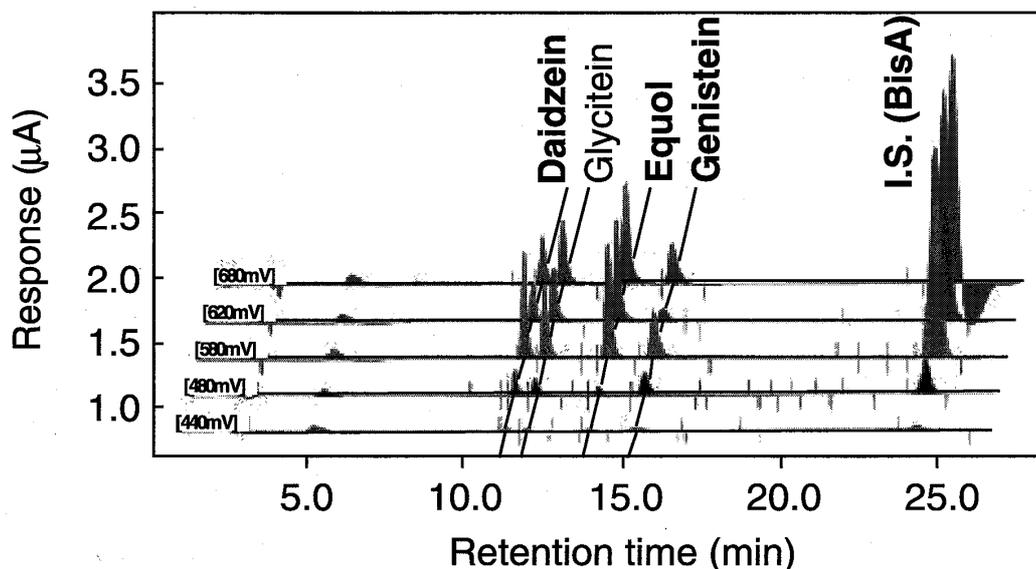


Figure 1. Multichannel chromatogram of a standard mixture. Shown is a typical chromatogram of a standard mixture containing 714.9 ng/ml of daidzein, 759.9 ng/ml of glycitein, 680.6 ng/ml of equol, 759.9 ng/ml of genistein, and 2.28 mg/ml bisphenol A (internal standard). The retention time for each isoflavone are as follows: daidzein, 10.48 min; glycitein, 11.14 min; equol, 13.18 min; genistein, 14.68 min; and bisphenol A, 23.68 min. The dominant channel (channel 6) is 580 mV, the leading channel (channel 5) is 480 mV, and the following channel (channel 7) is 620 mV.

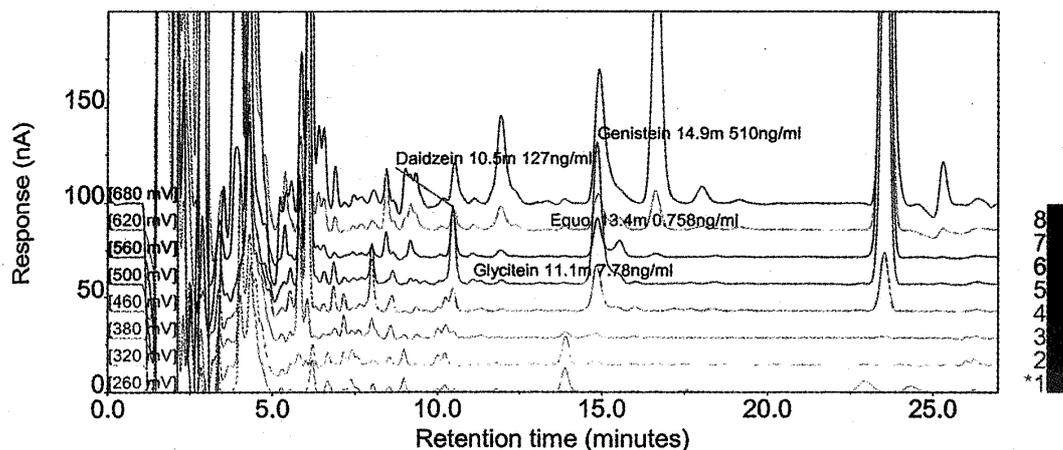


Figure 2. A typical chromatogram obtained from human plasma. Total isoflavones, both free and conjugated, were extracted from 200 μ l of human plasma. A 50- μ l aliquot of sample was loaded onto the chromatography column. The dominant channel (channel 6) is 580 mV. The signals of daidzein, equol, and genistein were identified by CoulArrayTM software (ESA Inc.).

&). The response ratios (leading [channel 5]/dominant [channel 6]) and (following [channel 7]/dominant [channel 6]) of a given cluster are specific for a particular compound. The ratios obtained from a pure standard can then be used to match a peak cluster of an unknown. The ratio accuracy is then calculated for the unknown as (leading [or following] response/dominant response from the unknown)/(leading [or following] response/dominant response from the standard). If the ratios are equivalent, the ratio accuracy value=1.

Identification of isoflavones in human serum

A Norman human serum sample was hydrolyzed by s-glucuronidase, extracted, separated by HPLC, and detected with the coulometric electrode (Fig. 2). Each isoflavone was identified by comparison with the retention times and the response profiles obtained from a standard (Fig. 1). The ratio accuracies for daidzein in this sample were 0.95 for channels 5/6 and 0.87 for channels 7/6; those for equol were 0.86 and 0.82; and those for genistein were 0.96 and 0.96. These ratio accuracies are all close to 1.0, verifying both the effectiveness and the qualitative utility of this system for detecting the three isoflavones in human serum samples. Although our electrode voltage settings were somewhat different from those described by Gamache and coworkers⁽²⁰⁾, our results suggest that they were effective for the simultaneous detection of daidzein, genistein, and equol in serum samples. Because the recovery test for glycitein alone was not sufficient enough for quantification in target measurement range, we did not use the data in these measurements.

Isoflavone standard curve

Next, we checked the quantitative limit for detection of isoflavones. In the current study, daidzein, genistein, and equol demonstrated the largest peak area at 580 mV. We found good linearity for the peak area at 580 mV and the quantity of each isoflavone, enabling the detection limit of each isoflavone to be determined. As shown in Figure 3, we obtained linear standard curves from 1.18 to 953.3 ng/ml of daidzein, 1.25 to 1013.0 ng/ml of genistein, and 1.12 to

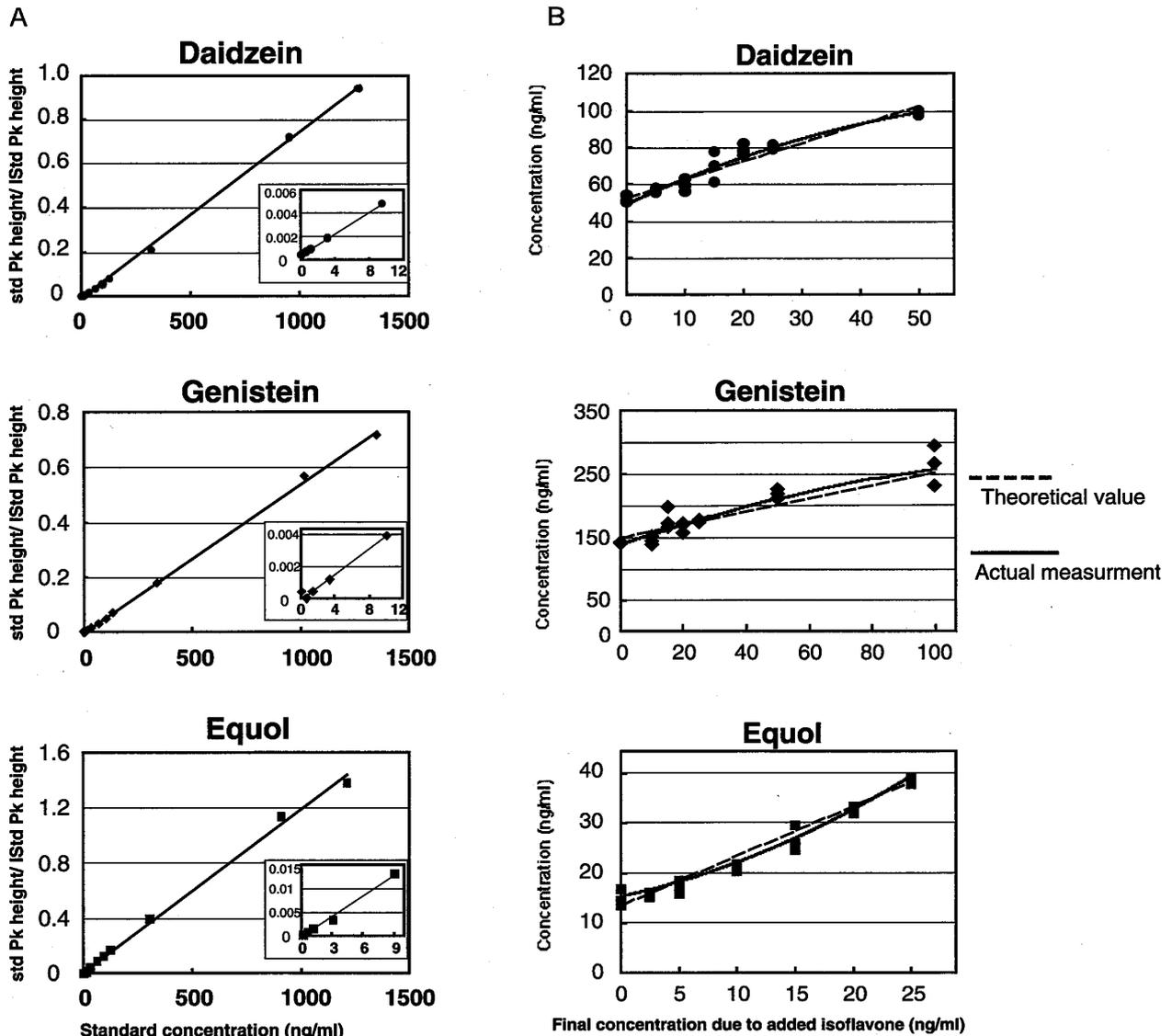


Figure 3 -A. Graphs displaying the standard curves of three isoflavones (daidzein, genistein, and equol). The X-axis shows that the peak height of each standard, normalized by the peak height of the internal standard (2.28 mg/ml of bisphenol A). The Y-axis shows the concentration of standard isoflavones (ng/ml). The concentrations of standards were 1.18 to 953.3 ng/ml for daidzein, 1.25 to 1013.0 ng/ml for genistein, and 1.12 to 907.5 ng/ml for equol. Standard curves for lower concentrations are shown in the inner panel of each graph.

Figure 3 -B. Recovery test for three isoflavones in the human plasma. The X-axis shows the final concentration due to isoflavones added (ng/ml) The Y-axis shows the serum isoflavone concentration that was actually measured (ng/ml). Dotted lines show the theoretical values. Solid lines show the values actually measured.

907.5 ng/ml equol.

Selection of internal standard

To adjust for differences in extraction efficiency and other errors in sample pretreatment as well as isoflavone recovery rates, we selected bisphenol A as an internal standard after examination of 13 candidate compounds, including α 18-OH estradiol, stereoisomer of β 18-OH estradiol, and diethylstilbestrol (DES). But DES as an internal standard was not appropriate to apply due to the long retention time⁽²²⁾. Bisphenol A demonstrated a strong and sharp signal

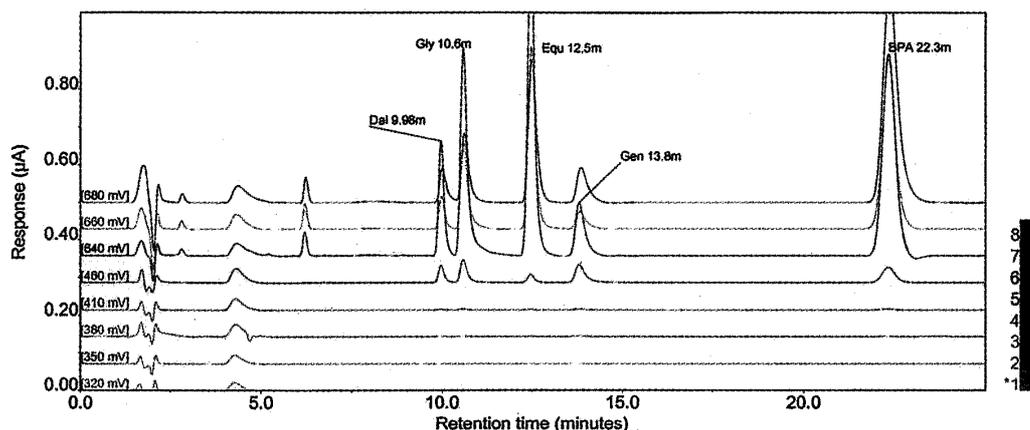


Figure 4. The typical chromatogram in recovery test with bisphenol A as an internal standard.

pattern and good linearity at 580 mV, and it is stable and gives a consistent retention time. Therefore, bisphenol A was added to the serum specimens as well as to the standard solutions as an internal standard. This analysis suggested that bisphenol A is appropriate for an internal standard.

Recovery experiment

To determine the extraction efficiency of each isoflavone, we spiked human serum samples with known amount of pure isoflavone and compared the actual measured and theoretical amounts. As shown in Figure 3-B and Figure 4, we found that the measured concentration of each isoflavone was quite similar to the theoretical values. Extraction efficiencies were 105.5% for daidzein, 126.6% for genistein, and 81.8% for equol. We also determined that the minimal concentration of each isoflavone that could be detected in human serum was 5.0 ng/ml for daidzein, 15.0 ng/ml for genistein, and 2.5 ng/ml for equol. These results suggest that our method of using bisphenol A as an internal standard is suitable for extracting all three isoflavones from human serum.

Validation in Soyaflavone E ingestion study

To validate this system with real experimental samples, we determined the serum isoflavone contents in healthy volunteers before and after ingestion of Soyaflavone E (isoflavone powder). This product is widely used as an ingredient to enrich isoflavones in various food products. Before the treatment, the concentrations of each isoflavone ranged from 17.4 to 162.5 ng/ml for daidzein, 47.1 to 563.0 ng/ml for genistein, and 2.8 to 50.1 ng/ml for equol (Table 2). The concentrations of isoflavones in the drum samples rose dramatically after three days of ingesting Soyaflavone E; the values ranged from 1092.5 to 3326.6 ng/ml for daidzein, 489.2 to 4271.9 ng/ml for genistein, and 2.0 to 335.4 ng/ml for equol. After Soyaflavone E treatment, the serum equol concentrations of two volunteers (M5 and F1) were quite low, although those of daidzein and genistein were quite elevated (Table 2). The daidzein, genistein, and equol levels were statistically different between before and after dosing ($p < 0.05$, X) by Student's t-test, respectively.

Table 2. Concentrations of three isoflavones in human serum before and after Soyafalvone E ingestions

	Before (ng/ml)			After (ng/ml)		
	Daidzein	Genistein	Equol	Daidzein	Genistein	Equol
A (M)	28.9	47.1	14.0	1301.6	573.3	323.4
B (M)	26.5	128.8	50.1	1092.5	786.3	335.4
C (M)	162.5	416.2	45.9	1760.0	771.9	162.9
D (M)	158.1	143.8	6.9	1744.4	489.2	38.0
E (M)	7.4	118.0	2.8	1500.2	658.4	2.5
F (F)	127.3	100.5	3.0	3326.6	895.9	8.4
G (F)	58.9	563.0	37.4	3322.3	4271.9	176.8

Plasma samples that were collected from volunteers before and 3 days after the treatment of soyaflavone E were analyzed. Total isoflavones were extracted from the plasma and analyzed by HPLC. (M) denotes a male volunteer, (F) denotes a female.

Discussion

Isoflavones in human urine were first quantified by gas chromatography (GC)^(22,23). Because the preparation required for GC is completed, a HPLC method using a reversed-phase column together with ultraviolet (UV) detection was developed for assessing isoflavones in bovine plasma and urine⁽²⁴⁾. Gamache et al.⁽²⁰⁾ also developed a way to detect isoflavones in human urine and rat plasma and tissue using reversed-phase HPLC coupled with coulometric array detection. The coulometric electrode has a high electrolytic efficiency; easily oxidized compounds can be detected by lower voltage electrodes, whereas compounds, which is relatively resistant to oxidation, can be detected with higher voltage electrodes. Based on the latter method, we are the first to use bisphenol A as an internal standard. Merits of bisphenol A use are stable, eluted as a sharp peak, and a reproducible retention time within 30 min. This allows correction of problem in precision caused by variations in injection volume or sample extraction efficiency. Moreover, this system expands the capabilities for multiple component analysis from a single injection and can minimize sample pre-purification steps when dealing with serum. Furthermore, because HPLC with coulometric detection could be cheaper and easier to initiate than GC-MS⁽²⁵⁾ and more efficient, sensitive, accurate and rapid than HPLC-UV detection, it would be used for large-scale human epidemiological studies of isoflavones in serum.

In the Soyafalvone E ingestion study, after intake of isoflavone powder, the concentrations of daidzein and genistein were substantially elevated (mean 6.5- and 39.6- fold, respectively). However, there were two separate populations of equol levels in seven individuals; in five individuals, equol concentrations rose markedly following ingestion, whereas the equol concentrations in the other two remained very low following ingestions. These results indicate that the concentrations of serum daidzein and genistein can be influenced by diet. However, for some populations, equol concentrations are affected by intestinal microflora rather than by the diet⁽²⁶⁾.

In conclusion, HPLC with coulometric array detector and bisphenol A as internal standard could efficiently detect daidzein, genistein and equol simultaneously in human serum. Using this method, we were able to identify equol producers in a general Japanese population. We propose that a rapid, accurate and sensitive analytical method for serum isoflavones is applicable to

large scale human epidemiological studies.

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高速液体クロマトグラフィー-クーロアレイ検出システムを用いた血清中イソフラボン測定方法

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要 約

目的：大豆イソフラボンの生理活性がヒトの健康に種々の影響を与えることを示す研究成果に大きな関心が集まっている。この研究に不可欠な血清中イソフラボン測定をクロメトリック電極アレイと高速液体クロマトグラフィーを用いて、迅速で再現性のいい測定方法を確立する。

方法：血漿サンプルは、抽出前に bisphenol A を内部標準として添加し、beta-glucuronidase/sulfatase で 3 時間 37°C で加水分解し、エタノール抽出後、20%メタノールで置換し、Binary gradient reversed-phase (C18) chromatography に注入し、酢酸ナトリウム緩衝液 (pH 4.8)-メタノール・アセトニトリルの移動層、流速 1.0 ml/min を用いて測定を行った。8 チャンネルの検出電極の内、3 チャ

ンネルを用いて、ダイゼイン、ジェネスタイン、エクオールのイソフラボン濃度について、ヒト血漿サンプルを用いて測定を繰り返し行った。

結果：3 種類のイソフラボンの測定回収率は、ダイゼイン 105.5%、ジェネスタイン 126.6%、エクオール 81.1% であり、定量限界はダイゼイン 5.0 ng/ml、ジェネスタイン 15.0 ng/ml、エクオール 2.5 ng/ml であり、bisphenol A を内部標準として加えることにより再現性が非常に向上した。7 名のヒトの血漿中イソフラボン濃度のレンジは、ダイゼイン 17.4-162.5 ng/ml、ジェネスタイン 47.1-563.0 ng/ml、エクオール 2.8-50.1 ng/ml であった。この測定方法の改良により、疫学調査にも十分使える測定法となった。

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