Rapid and Convenient Detection of Urinary Equol by Thin-Layer Chromatography

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Summary Equol, a metabolite of daidzein for some intestinal microflora, is known to retain highly estrogenic activity and is of wide interest in relation to human health. However, not all humans can produce equol. In this study, detection of urinary equol using thin-layer chromatography (TLC) was performed to distinguish between equol producers and non-producers. After 36 h of soy food intake, urine, collected from 7 volunteers, was hydrolyzed, purified by reverse phase silica gel column and applied to normal phase TLC. Consequently, equol was clearly separated from the urine samples and discriminated the equol producers in this system. The detection limit of equol was at least 20 ng.

Key Words equol, detection, urine, thin-layer chromatography

Isoflavonoids, which are mainly present at high concentrations in legumes such as soybeans, have weak estrogenic activities, and might be beneficial for prevention of breast (1, 2) or prostate (3, 4) cancer, osteoporosis (5) and menopausal syndromes (6). Owing to these features, soy isoflavonoids are popular as components of healthy foods or supplements. However, there seems to be inter-individual differences in the physiological activities of soy isoflavonoids.

It has been reported that intestinal microflora are involved in the metabolism of isoflavonoids and lead to the inter-individual differences observed. It was proposed that daidzein is transformed into dihydrodaidzein (DHD) and further metabolized into o-desmethylango- lensin (o-DMA) or equol (Fig. 1) (7, 8). Equol, one of the daidzein metabolites of intestinal microflora, is known to have higher estrogenic (9, 10) and antioxidant (11) activities than other major soy isoflavonoids (daidzein and genistein); however, not all humans can produce it. It is reported that 20–60% of healthy adults can produce equol (6, 12–16). Therefore, it might be beneficial to distinguish between equol producers and non-producers. These isoflavonoids, including equol, are absorbed in the intestine and finally excreted into urine through blood. Therefore, it is possible to identify equol producers by analyzing urine or plasma from a clinical sample.

Ordinarily, detection of equol from urine or plasma samples is carried out using high performance liquid chromatography (HPLC) (17), gas chromatography-mass spectrum (GC-MS) (1, 14, 18) or liquid chromatography (LC)-MS (3). However, these methods are not suitable for multiple sample-analyses such as discrimination of equol producers. In this study, we tried to establish an economical, rapid and convenient equol detection system for discriminating the producers using thin-layer chromatography (TLC).

MATERIALS AND METHODS

Chemicals and materials. The following reagents were used as standard substances: equol, E-5880 LC Laboratories (Woburn, MA, USA); daidzein, D2668 Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan); genistein, G6649 Sigma (St. Louis, MO, USA); dihydrodaidzein, D449000 Toronto Research Chemicals Inc. (North York, Canada). Beta-glucuronidase (EC 3.2.1.31) type H-2 from Helix pomatia was purchased from Sigma, which has two kinds of enzyme activity (98,000 U/mL of β-glucuronidase and 2,400 U/mL of sulfatase). Reverse phase silica gel (Wakogel 50C18) was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Silica gel 60 F254 and RP-18 F254s TLC plates were obtained from Merck KGaA (Darmstadt, Germany).

Urine collection. Urine samples were collected from 7 volunteers about 36 h after intake of soybean foods.

Sample preparation and extraction. Nine hundred microliters of urine was incubated overnight at 37°C with 5 mg ascorbic acid and 2 µL of β-glucuronidase (97.6 unit/µL) in 100 µL of 1.5 M sodium acetate (pH 4.1), and then each hydrolysate was applied onto the C18 column (200 µL) in 100 µL methanol. The column was washed with 500 µL water and three times with 500 µL 30% methanol and then eluted with 500 µL methanol. The eluent was dried under vacuum.

Detection of isoflavonoid species. The samples were dissolved in 10 µL methanol, and analyzed by normal phase and reverse phase TLC plate in a solvent system of toluene:acetone (2:1) and in a solvent system of
acetonitrile : water : acetic acid (60 : 40 : 1). The mobility of isoflavonoids, developed on TLC, was visualized with a UV transilluminator (312 nm). If necessary, the TLC plate was subjected to saturated iodine vapor for 1 min prior to UV visualization. The silica gel of the position, which corresponded to equol, was scratched and eluted with methanol. Then, the eluent was analyzed by an HPLC-photodiode array (PDA) detection system.

**Instruments.** An L-2130 equipped with L-2450 PDA and L-2300 column oven (Hitachi High-Technologies Corp., Tokyo, Japan) was used for HPLC analysis.

Data were analyzed using the attached software. The fractions were separated at 40°C on a Wakopak Navi C18-5 column (4.6×150 mm, Wako Pure Chemical Industries, Ltd.) using a one-step linear gradient. Mobile phase A [water : acetic acid (100 : 1)] and B [water : acetonitrile : acetic acid (50 : 50 : 1)] ratios where changed after 5 min from 7 : 3 to 3 : 7 for 30 min at a flow rate of 1.0 mL/min.

**RESULTS AND DISCUSSION**

Reverse phase column chromatography, connected
with an HPLC system, is generally used for isolation and identification of soy isoflavonoid (17). To begin with, the isolation of commercial soy isoflavonoid compound was performed using RP-18 reverse phase TLC. However, as shown in Fig. 2A, equol was hardly separated from genistein. In addition, daidzein could not be discriminated from DHD. However, these 4 compounds were separated in the normal phase TLC (Fig. 2B). Notably, the equol spot was gradually thickened by UV irradiation and visualized with brown color under visible light. The sensitivity was further enhanced by development in the presence of iodine vapor. In this assay system, the detection limit of equol was at least 20 ng (Fig. 2C).

Then, this method was applied to identify the equol-producing volunteers. Urine samples collected from 7 volunteers, 36 h after taking soybean foods (approximately 50 g as natto) were hydrolyzed with β-glucuronidase, purified by C18 reverse phase spin column chromatography and analyzed by normal phase TLC. Figure 3 shows that a spot corresponding to equol (RF=0.49) was detected in 3 of 7 volunteers (Fig. 3, lanes 3, 4 and 7). The peculiar colors of spots corresponding to daidzein (bright blue), dihydrodaidzein (dark blue) and genistein (dark gray, resemble to equol) were also observed on RF value of 0.29, 0.34 and 0.39, respectively. In order to confirm whether the compound corresponding to the spot was equol, the TLC-purified compound from one sample (Fig. 3, lane 3 sample) was reanalyzed by an HPLC-PDA detection system. The HPLC-PDA data clearly showed that the mono peak was observed at a retention time of 27.3 min with twin UV-absorption spectrum at 228 and 280 nm (Fig. 4A and B), which is consistent with the report of Wang et al. (19). Consequently, the HPLC profile at 280 nm of the purified compound also corresponded with that of com-

Fig. 3. Detection of isoflavonoid species from urine. The urine samples were treated as described in “Materials and Methods,” developed in toluene: acetone (2:1) and visualized with UV at 312 nm. Lane 1, equol and dihydrodaidzein standards (20 μg); lanes 2–8, urine samples (whole volume of the dried eluent); lane 9, equol and genistein standards (20 μg).

Fig. 4. HPLC-PDA analysis of purified equol from urine. Panel A, HPLC-PDA spectrum of purified equol from urine. Urine samples were separated by TLC, scratched and eluted with MeOH. Panel B, UV spectrum of purified equol. Panel C, overlaid HPLC chromatogram of purified equol and commercial equol standard.
mercial equol standard (Fig. 4C).

In our preliminary trial, no spot including equol was detected in the normal phase TLC system without β-glucuronidase treatment. Adlercreutz et al. reported that more than 60% of urinary isoflavonoids occurred as the monoglucuronide, and daidzein and equol, in particular, were excreted to a relatively high extent as sulfoglucurononides (20).

To search for an appropriate sample preparation, the time course of equol excretion was monitored with one volunteer’s urine by using the TLC system. The results suggested that urinary equol excretion is retained within the detectable level at 30 to 40 h after soybean food intake (data not shown).

There are several definitions to discriminate the equol producer. Some workers interpret a person excreting urinary equol at a concentration greater than 1,000 nmol/L (240 ng/mL) as an equol producer (I. 18, 21). Moreover, the production of equol could not be confirmed in the fecal culture system with daidzein (22) from ‘the equol non-producer’ discriminated in this assay, while it could be distinguished from that of ‘the producer’ (data not shown). On the other hand, the cutoff value of plasma equol is estimated to be lower than that for urine (20 ng/mL) (11). To confirm more practical sensitivity, 20 ng of commercial equol was mixed with the urine sample of the equol non-producer (Fig. 3, lane 2) and subjected to the TLC system. Consequently, equol was detected as an individual spot, which was compatible with the result of Fig. 2C (data not shown). In the present examination, we used urine as a clinical sample in view of non-invasive sample collection. This assay system might have enough sensitivity to be applied to plasma sample-assay. For quantitation of equol, a densitometric analysis of the TLC spot might be also available. In conclusion, the present TLC assay system might be adapted for distinction between equol producers and non-producers.

However, while HPLC or MS are popular tools for equol detection, they are relatively expensive and have troublesome operations. In addition, they are not adapted for multiple sample assays and take time to analyze per sample. In comparison, the TLC system is a rapid and convenient method to analyze multiple samples simultaneously in a short time.

Recently, the physiological effects of equol have been increasingly studied. It might be quite useful to know an individual constitution, whether someone is an equol producer or non-producer, to examine any association between the intake of soy foods or isoflavonoid supplements and human health. Furthermore, it is expected that this novel assay method represents significant progress in the studies for intestinal microflora, such as an application for screening of equol-producing bacteria.

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REFERENCES


