Journal of Pharmaceutical and Biomedical Analysis 50 (2009) 939–946

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Simultaneous determination of phytoestrogens and key metabolites in breast cancer patients' urine by liquid chromatography–tandem mass spectrometry

Jing Yu[a,b], Qian Wu[b], Shanlei Qiao[b], Zeping Yu[c], Nianzu Jin[b], Boyang Yu[a,*]

[a] Department of Chinese Medicinal Prescription, China Pharmaceutical University, Nanjing 210038, PR China
[b] Department of Hygienic Analysis and Detection, School of Public Health, Nanjing Medical University, Nanjing 210029, PR China
[c] Research Institute of General Surgery, Nanjing General Hospital of PLA, Nanjing Military Command, Nanjing 210002, PR China

ARTICLE INFO

Article history:
Received 9 February 2009
Received in revised form 10 June 2009
Accepted 11 June 2009
Available online 24 June 2009

Keywords:
Phytoestrogens
Liquid chromatography–tandem mass spectrometry
Urine
Breast cancer

ABSTRACT

A novel, selective and sensitive liquid chromatography–tandem mass spectrometry method has been developed and validated for the simultaneous determination of phytoestrogens and their key metabolites in human urine in this study. This method includes internal standard (IS) screening, analytical sample preparation procedure establishment, and linear range investigation. The analytical sample was extracted by liquid–liquid extraction from urine sample. The phytoestrogens and related key metabolites were separated with Agilent Zorbax Eclipse XDB-C18 chromatographic column using methanol and water as mobile phase. The Quattro premier MICROMASS mass spectrometer in negative ion selected reaction monitoring (SRM) mode using electrospray ionization was applied to detect the phytoestrogens and key metabolites. To validate the developed liquid chromatography–tandem mass spectrometry method, the intra- and inter-day precisions, specificity, sensitivity, reproducibility, and sample detective concentration range were evaluated. This is the first reported phytoestrogens analysis and validation study that demonstrates the feasibility of using liquid chromatography–electrospray ionization mass spectrometry to simultaneously analyze ten analytes including both phytoestrogens and their key metabolites in urine samples collected for epidemiological studies in human.

1. Introduction

Phytoestrogens are a group of chemical compounds that have been found in many plants, which consist mainly of three classes: isoflavones, lignans, and coumestans [1]. Of them, isoflavonoid phytoesreogens, such as genistein and daidzein, are primarily found in soy products and legumes [2], while lignans, such as secoisolariciresinol, are mostly found in the woody portions of fruit and vegetable, seed coats and grain bran of fiber-rich cereals [3]. Coumestans are less abundant in the diet and therefore are less well-studied [4].

The function of phytoestrogens is similar to that of estrogen, a hormone necessary for childbearing and involved with women bone and heart health. However, previous study suggests that higher exposure to estrogens over a lifetime increases breast cancer risk [5,6]. More than 1.2 million of women are diagnosed with breast cancer every year and approximately one third of women with estrogen receptor-positive breast cancer experience a recurrence [7]. As estrogen-like chemicals, phytoestrogens can act as mimics of estrogen in the human body, especially at low dose. At high doses, however, phytoestrogens may act differently from estrogen, such as eliciting estrogenic and antiestrogenic activities affecting cells communication pathways, preventing the formation of blood vessels to tumors, and so on. Phytoestrogens are thus being investigated for their roles in modulating breast cancer (BC) growth [8–10]. The metabolic activities of phytoestrogens involve very complicated enzymatic processes that occur in human liver, intestine and colon, which have been extensively studied [11,12]. Previous studies have shown that some metabolites such as equol, enterodiol and enterolactone derived from daizein and plant lignans are even more important for the biological activity of phytoestrogens [13–15]. Therefore, in addition to studying phytoestrogens, it is also important to determine the effects of related metabolites of phytoestrogens and analyze their metabolism levels.

In order to analyze a large amount of samples in epidemiological studies, it is highly essential to develop a reliable and reproducible assay. At the mean time, this assay must be also very sensitive to handle with small volume samples. Various analytical techniques [16–18] have been used to quantify the low levels of phytoestrogens present in biological fluids in previous studies. There has been increasing interest and popularity in the application of some advanced analytical techniques based on liquid chromatography

* Corresponding author at: Department of Traditional Chinese Prescription, China Pharmaceutical University, BoxC-09, Shennong Road, Nanjing 210038, PR China. Tel.: +86 25 85391042; fax: +86 25 83313080.
E-mail addresses: yujingfei@126.com (J. Yu), boyangyu59@163.com (B. Yu).

0731-7085/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2009.06.032

(LC) with tandem mass spectrometric (LC–MS/MS) detection in phytoestrogens, primarily due to the inherent selectivity and sensitivity of MS/MS detection [19–21]. Grace et al. [22] developed a high throughput method to titrate phytoestrogens using LC–MS/MS. In their method, the samples were extracted using 96-well plate and then analyzed incorporating column switching, which was expensive and complex, and required special instruments. Rybak et al. [23] compared the analytical performance of atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) for the quantitative determination of six urinary phytoestrogens. However, they did not report the determination of the actual samples.

In this work, we developed a convenient, selective and highly sensitive method that couples LC with ESI-MS/MS and employed it to determine the phytoestrogens and their key metabolites in human urine. The analytes include three isoflavones (e.g., daidzein, equol, and genistein), three lignans (e.g., secoisolariciresinol, enterodiol, and enterolactone), three flavanones (e.g., formononetin, naringenin, and biochanin A) and coumestrol. The chemical structures of these species are shown in Fig. 1. The developed phytoestrogens assay, including internal standard (IS) screening and evaluation, sample extraction and separation using chromatography, linear range determination, and analysis procedure using LC–MS/MS, was described in detail in this article. In addition, the precisions, the specificity, the sensitivity, the reproducibility, and the sample detection range of this assay were also evaluated and discussed. The results demonstrate that this method is efficient in analyzing large numbers of urine samples collected for epidemiological studies in human.

2. Experimental

2.1. Materials

β-Glucuronidase/sulfatase from *Helix pomatia* (Type HP-2, ≥500 Sigma units β-glucuronidase and ≤37.5 units sulfatase activity) and all the phytoestrogens and metabolites, daidzein, equol, genistein, formononetin, secoisolariciresinol, enterolactone, enterodiol, naringenin, biochanin A, coumestrol, and phenytoin, were obtained from Sigma (St. Louis, MO). Methanol was chromatographic pure grade and purchased from Merck (Merck Company, Germany). All other analytical grade chemical reagents were purchased from Sigma unless otherwise stated. The deionized water was distilled before using.

2.2. Samples

Overnight urinary samples were obtained from 141 female breast cancer cases and 141 female cancer-free controls. All samples (50 mL) were stored at −80 °C before using.

All subjects were genetically unrelated ethnic Han Chinese. All patient volunteers were diagnosed with breast cancer in the period of July 2006 and November 2007, according to the National Diagno-

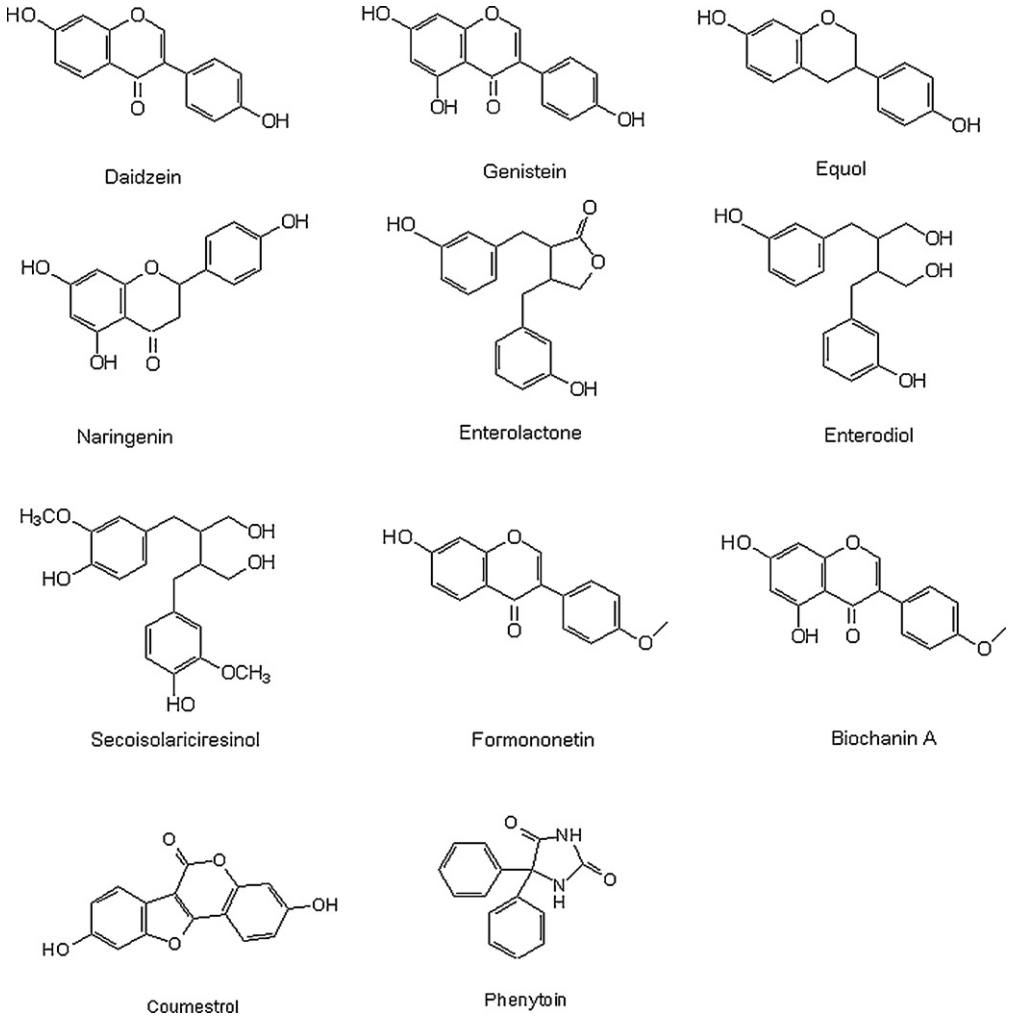

Fig. 1. Structures of the phytoestrogens analyzed and phenytoin (IS).

Table 1
Tandem mass spectrometry selected reaction monitoring conditions of ten analytes and phenytoin (IS).

Analyte	Molecular ion (*m*/*z*)	Product ion (*m*/*z*)	Dwell time (s)	Cone voltages (V)	Coll energy (eV)
Equol	241.0	121.0	0.1	20	11
Daidzein	253.0	224.0	0.1	30	26
Formononetin	267.0	252.0	0.1	30	20
Coumestrol	267.3	239.5	0.1	25	24
Genistein	269.0	133.0	0.1	30	36
Naringenin	271.2	151.2	0.1	30	19
Biochanin A	283.4	268.3	0.1	35	23
Enterolactone	297.3	253.4	0.1	30	20
Enterodiol	301.0	253.3	0.1	25	24
Secoisolariciresinol	361.4	165.3	0.1	30	25
Phenytoin	251.1	208.4	0.1	25	16

sis Standard for breast Cancer from the Nanjing General Hospital of Chinese PLA. There were no age, sex, and histology restrictions, but the patients with previous cancer history or unknown conditions of radiotherapy/chemotherapy were excluded. The cancer-free control subjects came from other clinics departments of the same hospital during the same period when the cases were recruited. They were outpatients in the clinics of general surgery, internal medicine, orthopedics, and otorhinolaryngology and without any hormone-related diseases. All controls were frequency matched to the cases by age (±5 years) and residential area (urban or countryside).

2.3. Instrumentations and operating conditions

Liquid chromatography was performed using a Waters 2695 HPLC system, which was coupled to a Quattro premier MICROMASS mass spectrometer with an electrospray ionization (ESI) interface. The whole analytical system was controlled using MassLynx 4.0 software.

The phytoestrogens and metabolites separations were carried out with Agilent Zorbax Eclipse XDB-C18 (5 μm, 150 mm × 2.1 mm) chromatographic column with temperature setpoint at 35 °C. The mobile phase consisted of methanol (solvent A) and water (solvent B) was set at a flow rate of 0.15 mL/min. A gradient program was used in elution step: the volume of solvent A was increased from 40% to 50% over 1 min and increased from 50% to 80% over the next 7 min. The column was flushed with 80% solvent A for 7 min and re-equilibrated with 40% solvent A for 15 min-running.

The eluate from HPLC system was fed into the MS/MS system directly for detection using selected reaction monitoring (SRM). The ESI source was set at negative ionization mode, with the capillary voltage at 2.5 kV and the sample cone temperature was maintained at 125 °C. Nitrogen was used as both desolvation gas with temperature of 350 °C and cone gas at flow rate of 600 and 100 L/h, respectively. Cone voltages and collision energies were optimized for each analyte during infusion of the pure standard and the most abundant fragment ion chosen for the selected reaction monitoring (SRM) transition. The detailed mass spectrometric operation conditions are summarized in Table 1. All HPLC–MS/MS components were quantified by interpolating peak area ratios for the MS/MS transitions against a calibration curve obtained from aqueous calibrators (1/*x* weighting).

2.4. Preparation of reference standard stock solutions, internal standard stock solutions and β-glucuronidase/sulfatase solutions

The primary reference standards and quality controls (QC) stock solutions of ten analytes (daidzein, equol, genistein, formononetin, secoisolariciresinol, enterolactone, enterodiol, naringenin, biochanin A, coumestrol) were prepared separately. The

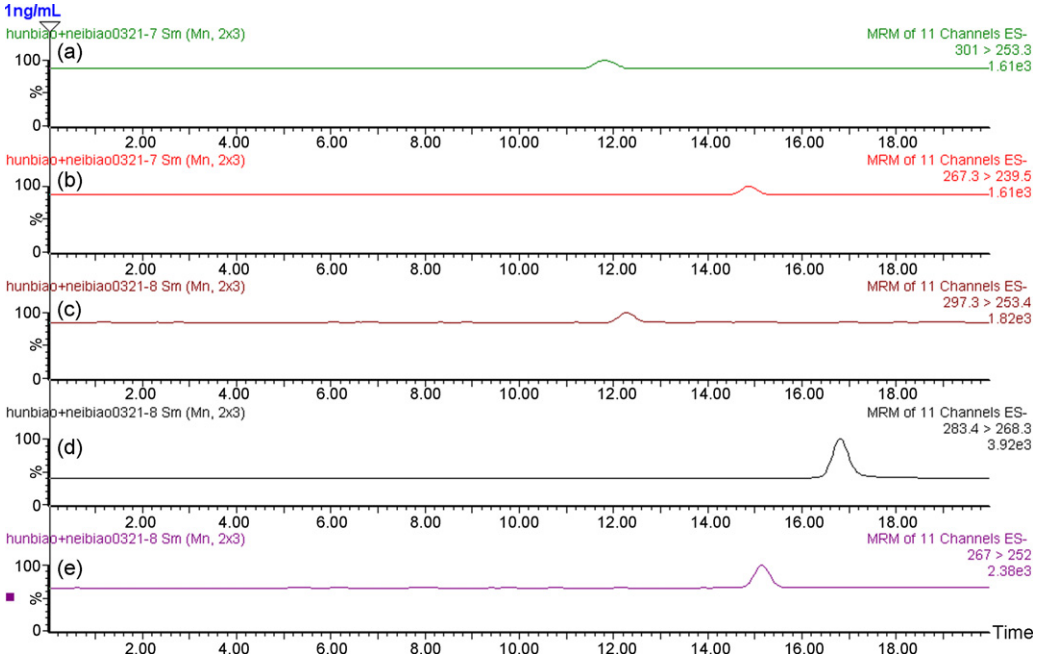

Fig. 2. Representative SRM chromatograms of phytoestrogens and key metabolites in human urine sample: (a) enterodiol; (b) coumestrol; (c) enterolactone; (d) biochanin A; (e) formononetin.

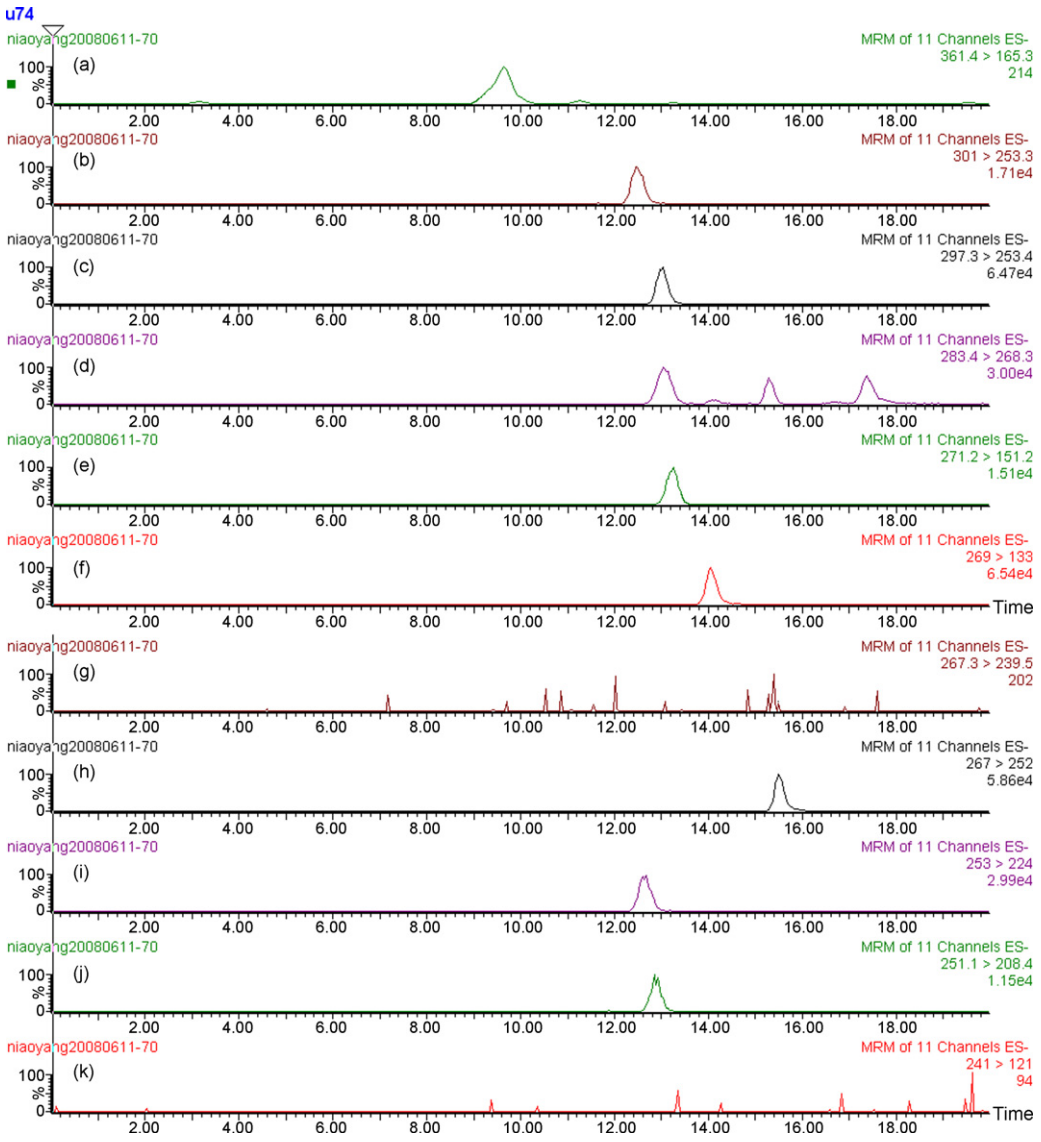

Fig. 3. SRM chromatograms of phytoestrogens and key metabolites, and phenytoin (IS) in a cancer patient urine sample: (a) secoisolariciresinol; (b) enterodiol; (c) enterolactone; (d) biochanin A; (e) naringenin; (f) genistein; (g) coumestrol; (h) formononetin; (i) daidzein; (j) phenytoin and (k) equol.

primary stock solutions were prepared by dissolving in methanol at 500 μg/mL. All these solutions were stored at 4 °C. The stock solution of phenytoin (IS) was prepared in methanol at a concentration of 1 mg/mL and was stored at 4 °C.

A 20 ku/mL of stock solution of β-glucuronidase/sulfatase in 1 mol/L ammonium acetate buffer (pH 5.0) was prepared and used freshly to enzymatically deconjugate the analytes of glucuronide and sulfate moieties during sample preparation.

2.5. Calibration curves

All analytes were quantified by means of calibration curves generated from analyte reference standards mixture with known concentrations. Calibration curve samples were prepared by spiking 0.2 mL of synthetic urine with appropriate reference standard stock solution (see Section 2.4) together with constant levels of IS (200 ng/mL), producing the calibration curve internal concen-

Table 2
Dynamic range of ten analytes ($n = 5$).

Analyte	Dynamic range (ng/mL)	Mean equation $y = ax + b$	Correlation coefficients	LOD (ng/mL)
Equol	5–2000	$y = 0.0018x - 0.0895$	0.9991	0.2
Daidzein	5–2000	$y = 0.0024x + 0.0945$	0.9941	0.2
Formononetin	0.5–2000	$y = 0.0537x + 2.8557$	0.9976	0.01
Coumestrol	1–2000	$y = 0.0037x + 0.3175$	0.9965	0.1
Genistein	5–2000	$y = 0.0048x + 0.1793$	0.9965	0.2
Naringenin	5–2000	$y = 0.0434x - 0.0470$	0.9975	0.2
Biochanin A	0.5–2000	$y = 0.0162x + 0.49025$	0.9979	0.01
Enterolactone	0.5–2000	$y = 0.0045x - 0.2522$	0.9974	0.01
Enterodiol	1–2000	$y = 0.0085x - 0.1726$	0.9992	0.1
Secoisolariciresinol	20–2000	$y = 0.0521x - 0.3546$	0.9970	2.0

tration points equivalent to 0.5, 1, 5, 20, 50, 100, 200, 500, 1000 and 2000 ng/mL. In each run, a synthetic urine sample (no IS) was also analyzed to confirm the absence of interferences. All calibration curves were generated by determining the best-fit of peak area ratios (peak area of analyte/peak area of internal standard) versus concentrations and fitted to the equation $R = bx + a$.

2.6. Preparation of quality control samples

Quality control samples were prepared daily by spiking 0.2 mL synthetic urine with proper volume of corresponding standard solution to produce a final concentration of 20, 200 and 1000 ng/mL of ten analytes, respectively, and a final concentration of 200 ng/mL of internal standard.

2.7. Preparation of samples

Quality control, calibration curve and urine samples were extracted employing a liquid–liquid extraction technique. Urine (200 μL) was thawed at room temperature before adding 200 μL 1 mol/L ammonium acetate buffer (pH5.0) and 10 μL phenytoin (4.0 μg/mL) as internal standard (IS), and 50 μL β-glucuronidase/sulfatase solution (20 ku/mL). Conjugates were allowed to be hydrolyzed to aglycones at 37 °C overnight. 2.0 mL acetic acid ethyl ester was added and the mixture was vortexed for 2 min. The organic layer was evaporated under a stream of nitrogen at 40 °C. The residue was reconstituted with 200 μL mobile phase and an aliquot of 10 μL of sample was injected into the LC–MS/MS system.

2.8. Method validation

The specificity of this method was investigated by analyzing synthetic urine samples. Each synthetic urine sample was tested for interference using the proposed extraction procedure and chromatographic/mass spectroscopic conditions, and was compared with those obtained with an aqueous solution of the analyte at a concentration near the lower limit of quantification (LLOQ).

The matrix effect on the ionization of the analytes was evaluated by comparing the peak areas of the analytes resolved in the blank sample (the final solution of synthetic urine after extraction and reconstitution) with that resolved in the mobile phase. Three different concentrations of ten phytoesreogens (20, 200 and 1000 ng/mL) and 200 ng/mL of the IS were evaluated by analyzing five samples at each level. If the ratio is <85% or >115%, an exogenous matrix effect is implied.

Calibration curves of the concentrations of ten analyses ranging from 0.5 to 2000 ng/mL were extracted and assayed. Synthetic urine samples were analyzed to confirm the absence of interferences but not used to construct the calibration function.

The precision of the assay was determined from the QC urine samples by replicate analyses of three concentration levels of ten analyses (20, 200 and 1000 ng/mL). Intra-day precision and accuracy were determined by repeated analyses of the group of standards on 1 day ($n = 5$). Inter-day precision and accuracy were determined by repeated analyses on three consecutive days ($n = 5$ series per day). The concentration of each sample was determined using the calibration curve prepared and analyzed on the same day.

The extraction recoveries of ten analyses were determined at low, medium and high concentrations. Recovery rates were calcu-

Table 3
The intra- and inter-day precision, the accuracy of the method for determination of ten analytes (intra-day: $n = 5$; inter-day: $n = 5$ series per day × 3 days).

Analyte	Added concentration (ng/mL)	Intra-day			Inter-day		
		Detected conc. (mean ± S.D., ng/mL)	Precision (%)	Accuracy (%)	Detected conc. (mean ± S.D., ng/mL)	Precision (%)	Accuracy (%)
Equol	20	22.28 ± 2.50	11.2	111.40	22.96 ± 3.07	13.4	114.80
	200	213.51 ± 16.35	7.7	106.76	218.84 ± 16.72	7.6	109.42
	1000	995.23 ± 41.79	4.2	99.52	991.13 ± 43.28	4.4	99.11
Daidzein	20	22.70 ± 2.22	9.8	111.35	22.98 ± 2.43	10.5	114.90
	200	189.32 ± 16.09	8.5	94.66	192.32 ± 16.35	8.5	96.16
	1000	1021.55 ± 52.10	5.1	102.16	1034.86 ± 53.46	5.2	103.49
Formononetin	20	18.43 ± 1.34	7.3	92.15	18.62 ± 1.56	8.4	93.10
	200	210.84 ± 13.07	6.2	105.42	212.54 ± 13.46	6.3	106.27
	1000	988.33 ± 30.64	3.1	98.83	990.13 ± 30.98	3.1	99.01
Coumestrol	20	22.12 ± 1.57	7.1	110.06	22.23 ± 1.61	7.1	110.15
	200	209.47 ± 15.08	7.2	104.74	210.12 ± 15.50	7.4	105.06
	1000	1023.12 ± 29.67	2.9	102.31	1033.32 ± 35.08	3.4	103.33
Genistein	20	21.96 ± 2.15	9.8	109.80	21.98 ± 2.46	11.2	109.90
	200	219.78 ± 16.27	7.4	109.89	221.92 ± 15.39	6.9	110.96
	1000	975.31 ± 39.01	4.0	97.53	975.34 ± 39.18	4.0	97.53
Naringenin	20	18.05 ± 1.98	11.0	90.25	18.74 ± 2.02	10.8	93.70
	200	229.34 ± 19.05	8.3	114.67	227.57 ± 19.13	8.2	113.68
	1000	963.69 ± 46.86	4.8	96.27	988.45 ± 57.99	5.8	98.84
Biochanin A	20	17.18 ± 1.70	9.9	85.90	17.59 ± 1.85	10.5	87.95
	200	185.37 ± 12.05	6.5	92.69	190.23 ± 12.10	6.4	95.12
	1000	1041.11 ± 44.77	4.3	104.11	1052.35 ± 54.98	5.2	105.23
Enterolactone	20	18.13 ± 2.05	11.3	90.65	18.76 ± 2.37	12.6	93.80
	200	224.56 ± 18.41	8.2	112.28	219.61 ± 21.56	9.8	109.80
	1000	1032.23 ± 52.64	5.1	103.22	1018.68 ± 62.47	6.1	101.87
Enterodiol	20	17.01 ± 1.74	10.2	85.05	17.85 ± 2.23	12.5	89.25
	200	218.26 ± 13.53	6.2	109.13	220.35 ± 13.70	6.2	110.17
	1000	1012.84 ± 49.63	4.9	101.28	1023.32 ± 49.16	4.8	102.33
Secoisolariciresinol	20	22.57 ± 2.29	10.1	112.85	22.60 ± 2.33	10.3	113.00
	200	178.25 ± 13.37	7.5	89.13	185.42 ± 15.71	8.5	92.71
	1000	986.76 ± 35.52	3.6	98.68	990.05 ± 36.64	3.7	99.00

lated by comparing the analyte/IS peak area ratios obtained from extracted urine samples with those from the standard solutions at the same concentration.

Stability experiments were performed to evaluate the stability of ten analyses in stock solution and in urine samples under different temperature and timing conditions. The freeze and thaw stability was performed by testing three concentration levels of QC urine samples after three freeze (−80 °C) and thaw (room temperature) cycles. The short-term temperature stability was evaluated by determining QC urine samples at three concentration levels, which were kept at room temperature for a period that exceeded the routine preparation time of the samples (around 8 h). The long-term stability was assessed by analyzing QC urine samples at three concentration levels kept at low temperature (−80 °C) for 20 days. The post-preparative stability was measured by reanalyzing extracted QC samples kept under the autosampler conditions (4 °C) for 48 h. The stability of ten phytoesreogens and the IS working solutions were evaluated at room temperature for 8 h.

Standard curves produced in each analytical run were used to calculate the concentrations of ten analyses in the unknown samples. The reference standard samples were prepared along with the unknown samples in the same batch and analyzed in the middle of the run. The QC samples of ten analyses in five duplicates at three concentrations (20, 200 and 1000 ng/mL) were prepared and analyzed together with processed samples at intervals in each batch.

2.9. Statistical analysis

All experimental data were analyzed using STATA v7.0 software and presented as means ± standard deviation (S.D.). All P values are two-sided, and statistical significance was determined at $P < 0.05$.

3. Results and discussion

3.1. Internal standard (IS) selection

It is very important to select a suitable IS in order to obtain accurate results when MS/MS is used as the detector, as matrix effects may adversely impact the performance of the analysis. Phenytoin was finally selected as the internal standard in this study, since it has similar structure, retention action, ionization and extraction with ethyl acetate as compared with the phytoestrogen analytes. In addition, it has the less endogenous interference at m/z 251.1 than other analytes. The structure of phenytoin is shown in Fig. 1.

3.2. Urine sample hydrolysis and extraction

Despite many pharmacokinetics studies of the phytoestrogens [24,25], the actual types of conjugates circulating in the body and the position(s) of conjugation sites on the flavone skeleton are still unclear. In general, conjugated compounds in biological fluids are evaluated by measuring the free aglycones obtained after selective enzymatic hydrolysis. To prepare the analytical sample, conjugated compounds in human urine samples were hydrolyzed using β-glucuronidase, and the phytoestrogens were selectively extracted from any interfering components present within the matrix. As reported in previous studies [26,27], the extraction of phytoestrogens from biological fluids often involves complex sample preparation techniques containing multiple extraction steps. Liquid–liquid extraction was selected and used in this study because this technique could purify and concentrate samples effectively and easily. Different extraction reagents such as diethyl ether, ethyl acetate and n-hexane-isopropanol (95:5, v/v) were tested, evaluated and compared. The results show that ethyl acetate had high extraction efficiency and the extracted endogenous compounds did not interfere, suggesting that ethyl acetate was a suitable extraction reagent.

3.3. Analytes separation and assay specificity

Various analytes were separated using HPLC chromatographic column from the previous liquid–liquid extraction sample. Negative ion selected reaction monitoring (SRM) using electrospray ionization was applied to detect the phytoestrogenates in Quattro premier MICROMASS mass spectrometer.

Fig. 2 shows the five representative SRM (−) chromatograms from supplemented synthetic urine, and Fig. 3 shows the SRM (−) chromatograms of a cancer patient's urine. The total HPLC–MS/MS analysis time was 30 min per sample. It is apparent that the combination of HPLC with ESI-MS/MS led to short retention time and yielded both high selectivity and sensitivity. No interferences of the analytes were observed in the assay, implying the high selectivity of the SRM technique. There was no ion suppression effect in the established sample preparation under designed chromatographic conditions.

3.4. Sensitivity and dynamic range

As the concentrations of phytoestrogens varied significantly from sample to sample, it was important to ensure that the assay can be performed over a wide dynamic range. The desired dynamic ranges for the ten analytes are summarized in Table 2. The calibration curves of all ten analytes were analyzed and evaluated, and the linearity ranges were defined as 0.5 to 2000 ng/mL for formononetin, biochanin A and enterolactone, 1 to 2000 ng/mL for

Table 4
Recoveries of ten analytes from human urine ($n = 5$).

Analyte	Added concentration (ng/mL)	Recovery (mean ± S.D., %)	R.S.D. (%)
Equol	20	89.9 ± 10.22	11.3
	200	105.32 ± 8.91	8.4
	1000	95.64 ± 4.97	5.1
Daidzein	20	90.8 ± 11.63	12.8
	200	91.77 ± 6.32	6.9
	1000	101.21 ± 3.24	3.2
Formononetin	20	87.5 ± 9.88	11.2
	200	93.6 ± 7.47	7.9
	1000	95.2 ± 5.21	5.5
Coumestrol	20	115.32 ± 10.96	9.5
	200	110.17 ± 8.55	7.7
	1000	95.36 ± 4.17	4.3
Genistein	20	89.46 ± 12.54	14.0
	200	91.23 ± 7.83	8.5
	1000	93.57 ± 3.62	3.8
Naringenin	20	112.51 ± 14.11	12.8
	200	109.63 ± 8.49	7.7
	1000	103.47 ± 5.47	5.2
Biochanin A	20	113.14 ± 10.98	9.6
	200	106.24 ± 6.75	6.3
	1000	96.37 ± 4.84	5.0
Enterolactone	20	88.84 ± 12.05	13.5
	200	107.33 ± 7.83	7.3
	1000	103.71 ± 4.11	4.0
Enterodiol	20	111.12 ± 10.90	9.8
	200	94.54 ± 7.25	7.6
	1000	95.23 ± 3.43	3.6
Secoisolariciresinol	20	89.95 ± 11.12	12.4
	200	106.77 ± 8.31	7.8
	1000	102.29 ± 5.09	5.0

coumestrol and enterodiol, 5 to 2000 ng/mL for daidzein, equol, genistein, and naringenin, and 20 to 2000 ng/mL for secoisolariciresinol, respectively. The linear relationships between peak area ratio and concentrations of ten analytes were analyzed using linear regression analysis and shown in Table 2. The weighting factors of ten analytes were $1/x$.

In the next step, the limit of detection (LOD) was determined as concentration with a signal-to-noise ratio of 3:1. As shown in Table 2, the formononetin, biochanin A and enterolactone had LODs below 10 pg/mL, and daidzein, equol, genistein, naringenin had LODs of approximately 200 pg/mL. The LODs of enterodiol and coumestrol were below 100 pg/mL, and the LODs of secoisolariciresinol were 2 ng/mL because of its special structure (Fig. 1).

3.5. Quality control and reproducibility

The QC intra- and inter-day precision and the accuracy results of the ten analytes at concentrations of 20, 200, 1000 ng/mL are presented in Table 3. The intra-day precision ranges from 2.9% to 11.3%, while the inter-day precision ranges from 3.1% to 13.4%. The intra-day accuracy ranges from 85.05% to 114.67%, while the inter-day accuracy ranges from 87.95% to 114.90%.

The extraction recoveries determined for ten analytes were found to be consistent, precise and reproducible. The mean recoveries of the low, middle and high QC levels and their precisions are shown in Table 4.

3.6. Sample stability analysis

Table 5 summarizes the results of the short-term stability, the long-term stability, the post-preparative stability, and the freeze and thaw stability of the ten phytoesreogens. The stability data demonstrates the reliable stability behavior of the ten analytes under the testing-condition. Based on the data obtained, the working solutions of ten analytes and the IS are intact within 8 h.

3.7. Detection concentration ranges of phytoestrogens in urine samples

Table 6 shows the levels of phytoestrogens in the urine of women diagnosed with invasive breast cancer ($n = 141$) and cancer-free (control) ($n = 141$). Samples with concentrations outside the linear range of the calibration chart were successfully analyzed with dilution by an appropriate amount of mobile phase. These results demonstrated that the variation between each sample's phytoestrogens excretion levels was pretty large. The detection concentration ranges indicated that some analytes in several individual samples had very high concentrations. These high-concentration samples could skew the mean concentration to a higher value. Therefore, median concentrations are also shown in Table 6.

The results show that the most abundant phytoestrogen was isoflavones and the sum of median isoflavones concentrations was over five times higher than that of lignans in all samples. This is consistent with the literature statement that isoflavones intake is much more popular than lignans in the Chinese diet unlike Western countries [28]. Mean concentrations of equol, daidzein,

Table 5
Stability of ten analytes in human urine ($n = 5$).

Analyte	Added concentration (ng/mL)	Accuracy (mean ± S.D., %)			
		Short-term stability (8 h, room temperature)	Freeze and thaw stability (3cycles, −80 °C, room temperature)	Long-term stability (20 days, −80 °C)	Post-preparative stability (48 h, 4 °C)
Equol	20	90.50 ± 10.24	114.71 ± 11.33	88.58 ± 11.89	89.65 ± 10.87
	200	106.55 ± 7.56	108.90 ± 8.01	95.12 ± 9.56	105.18 ± 8.42
	1000	95.38 ± 4.13	97.25 ± 4.53	94.57 ± 4.60	103.25 ± 4.50
Daidzein	20	90.85 ± 9.53	108.13 ± 10.51	89.58 ± 15.77	112.43 ± 14.56
	200	91.01 ± 7.99	93.27 ± 8.49	86.25 ± 10.81	91.88 ± 8.15
	1000	101.72 ± 5.31	106.44 ± 6.05	110.73 ± 6.94	106.29 ± 5.06
Formononetin	20	87.46 ± 9.88	89.29 ± 10.14	90.44 ± 11.58	91.75 ± 8.79
	200	93.01 ± 6.19	108.73 ± 7.55	105.59 ± 9.72	90.05 ± 5.71
	1000	96.23 ± 4.96	102.43 ± 5.08	103.80 ± 6.33	95.17 ± 6.40
Coumestrol	20	113.69 ± 10.70	115.47 ± 12.15	111.39 ± 12.85	115.42 ± 11.51
	200	106.58 ± 6.81	106.94 ± 7.72	105.26 ± 6.47	107.05 ± 6.93
	1000	95.12 ± 5.02	96.83 ± 4.98	97.12 ± 4.33	96.70 ± 4.54
Genistein	20	89.49 ± 10.34	88.57 ± 11.69	86.97 ± 12.41	90.59 ± 12.82
	200	91.73 ± 5.07	107.35 ± 6.08	109.78 ± 5.82	93.24 ± 5.99
	1000	93.66 ± 4.88	94.71 ± 4.24	97.33 ± 5.56	95.75 ± 6.42
Naringenin	20	112.25 ± 11.16	115.88 ± 13.64	110.41 ± 12.08	92.29 ± 11.73
	200	109.87 ± 7.91	114.19 ± 7.56	112.39 ± 6.45	115.08 ± 8.48
	1000	103.77 ± 5.09	104.34 ± 6.40	105.44 ± 5.93	95.07 ± 4.98
Biochanin A	20	112.36 ± 10.44	114.55 ± 11.81	110.34 ± 11.77	111.49 ± 9.68
	200	107.00 ± 6.43	105.27 ± 7.06	104.18 ± 8.42	93.37 ± 6.15
	1000	97.98 ± 3.97	95.16 ± 4.70	105.61 ± 3.95	105.90 ± 4.27
Enterolactone	20	110.52 ± 10.80	89.71 ± 11.94	112.35 ± 10.63	87.73 ± 10.92
	200	94.56 ± 7.45	108.42 ± 8.78	110.07 ± 8.14	89.19 ± 8.03
	1000	103.97 ± 5.17	104.38 ± 4.99	97.42 ± 5.09	101.21 ± 4.34
Enterodiol	20	86.08 ± 8.95	89.15 ± 10.37	84.74 ± 11.58	85.39 ± 10.72
	200	109.97 ± 5.94	111.19 ± 6.88	114.21 ± 6.13	113.45 ± 7.77
	1000	95.84 ± 4.38	96.35 ± 4.76	93.28 ± 5.06	94.76 ± 5.28
Secoisolariciresinol	20	90.59 ± 10.86	88.61 ± 10.37	90.07 ± 12.23	89.45 ± 10.44
	200	106.27 ± 7.80	106.92 ± 8.11	95.58 ± 6.95	94.06 ± 7.31
	1000	103.43 ± 3.94	97.26 ± 4.02	96.20 ± 4.37	95.88 ± 5.08

Table 6
Mean, median, standard deviation, rang for ten analytes in human urine samples (ng/mL).

	Equol	Daidzein	Formononetin	Coumestrol	Genistein	Naringenin	Biochanin A	Enterolactone	Enterodiol	Secoisolariciresinol
Breast cancer patients (n = 141)										
Mean	396	1445	6	3.5	717	207	66	331	230	31
Median	0	518	3	0	211	63	49	70	62	0
Standard deviation	1428	2513	12	10	1313	424	68	724	439	67
Range	0–8347	0–12574	0–88	0–66	0–11674	41–2983	0–501	0–1895	0–2564	0–342
Health female volunteers (n = 141)										
Mean	228	1033	5	5	508	141	43	207	413	30
Median	0	415	7	0	228	106	38	134	45	0
Standard deviation	944	1727	10	7	1081	104	23	263	974	48
Range	0–8335	0–10377	0–56	0–79	0–5196	43–1020	0–179	0–962	0–4112	0–242

genistein, naringenin, biochanin A and enterolactone in the breast cancer patients' urine are higher than those of cancer-free controls. The mean concentration of enterodiol in patients' urine, on the contrary, is lower than that of cancer-free controls. However, no apparent correlation with breast cancer risk was detected for total urinary phytoestrogens between patients and cancer-free controls ($P > 0.05$) (data not shown).

4. Conclusion

The creative analytical method developed in this study provides a sensitive, precise, and highly specific assay for sub-ng/mL concentrations of phytoestrogens and key metabolites in human urine. This method has simple liquid–liquid extraction procedure and short run time, which can significantly shorten the total assay time. This is very important for large scale sample analysis. In fact, we have successfully applied this method to the simultaneous analysis of ten analytes in the urine samples collected for epidemiological studies in humans. The levels of phytoestrogens from the urine of women diagnosed with invasive breast cancer were compared with those from cancer-free females. These results can improve the understanding of the relationship between phytoestrogens and breast cancer.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (30572133) and the Foundation of Jiangsu Educational Committee (08KJB360003).

References

[1] L.U. Thompson, B.A. Boucher, Z. Liu, M. Cotterchio, N. Kreiger, Nutr. Cancer 54 (2006) 184–201.
[2] N.J. Turner, B.M. Thomson, I.C. Shaw, Nutr. Rev. 61 (2003) 204–213.
[3] T. Cornwell, W. Cohick, I. Raskin, Phytochemistry 65 (2004) 995–1016.
[4] M.S. Kurzer, X. Xu, Annu. Rev. Nutr. 17 (1997) 353–381.
[5] L. Adly, D. Hill, M.E. Sherman, S.R. Sturgeon, T. Fears, C. Mies, R.G. Ziegler, R.N. Hoover, C. Schairer, Int. J. Cancer 119 (2006) 2402–2407.
[6] H. Seeger, D. Wallwiener, A.O. Mueck, Gynecol. Endocrinol. 24 (2008) 576–579.
[7] E. Kubista, Wien. Med. Wochenschr. 151 (2001) 548–551.
[8] S.M. Mense, T.K. Hei, R.K. Ganju, H.K. Bhat, Environ. Health Perspect. 116 (2008) 426–433.
[9] S. Rice, S.A. Whitehead, Endocr. Relat. Cancer 3 (2006) 995–1015.
[10] H. Adlercreutz, Lancet Oncol. 3 (2002) 364–373.
[11] X. Liu, V.H. Tam, M. Hu, Mol. Pharm. 4 (2007) 873–882.
[12] S. Gredel, C. Grad, G. Rechkemmer, B. Watzl, Food Chem. Toxicol. 46 (2008) 3691–3696.
[13] K.D. Setchell, N.M. Brown, E. Lydeking-Olsen, J. Nutr. 132 (2002) 3577–3584.
[14] G.G. Kuhnle, C. Dell'Aquila, S.M. Aspinall, S.A. Runswick, A.A. Mulligan, S.A. Bingham, J. Agric. Food Chem. 56 (2008) 10099–10104.
[15] M. Hedelin, A. Klint, E.T. Chang, R. Bellocco, J.E. Johansson, S.O. Andersson, S.M. Heinonen, H. Adlercreutz, H.O. Adami, H. Grönberg, K.A. Bälter, Cancer Causes Control 17 (2006) 169–180.
[16] W. Wang, Proc. Soc. Exp. Biol. Med. 217 (1998) 281–287.
[17] E.J. Oliveira, D.G. Watson, J. Chromatogr., B: Biomed. Sci. Appl. 764 (2001) 3–25.
[18] C.L. Holder, M.I. Churchwell, D.R. Doerge, J. Agric. Food Chem. 47 (1999) 3764–3770.
[19] X. Chen, F. Qiu, D. Zhong, X. Duan, C. Liu, Pharmazie 60 (2005) 334–338.
[20] Z. Kuklenyik, X. Ye, J.A. Reich, L.L. Needham, A.M. Calafat, J. Chromatogr. Sci. 42 (2004) 495–500.
[21] L.D. Williams, N.C. Twaddle, M.I. Churchwell, D.R. Doerge, J. AOAC Int. 89 (2006) 1168–1173.
[22] P.B. Grace, N.S. Mistry, M.H. Carter, A.J. Leathem, P. Teale, J. Chromatogr., B: Anal. Technol. Biomed. Life Sci. 853 (2007) 138–146.
[23] M.E. Rybak, D.L. Parker, C.M. Pfeiffer, J. Chromatogr., B: Anal. Technol. Biomed. Life Sci. 861 (2008) 145–150.
[24] K. Hosoda, T. Furuta, A. Yokokawa, K. Ogura, A. Hiratsuka, K. Ishii, Drug Metab. Dispos. 36 (2008) 1485–1495.
[25] M.L. Antonelli, A. Faberi, E. Pastorini, R. Samperi, A. Laganà, Talanta 66 (2005) 1025–1033.
[26] D. Locati, S. Morandi, A. Cupisti, L. Ghiadoni, A. Arnoldi, Rapid Commun. Mass Spectrom. 19 (2005) 3473–3481.
[27] N.C. Twaddle, M.I. Churchwell, D.R. Doerge, J. Chromatogr., B: Anal. Technol. Biomed. Life Sci. 777 (2002) 139–145.
[28] H. Ward, G. Chapelais, G.G. Kuhnle, R. Luben, K.-T. Khaw, S. Bingham, Breast Cancer Res. 10 (2008) R32.