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A validated method for the quantification of enterodiol and enterolactone in plasma using isotope dilution liquid chromatography with tandem mass spectrometry

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Abstract

Enterolactone and enterodiol are phytoestrogens with structural similarity to endogenous estrogens. Because of their biological activities, they may affect the development of several diseases. To quantify enterodiol and enterolactone in plasma, we developed and validated a liquid chromatography–tandem mass spectrometry method with electrospray ionization using $^{13}C_3$ labeled isotopes. The method consists of a simple enzymatic hydrolysis and ether extraction followed by a rapid LC separation (run-time of 11 min). Detection limits as low as 0.15 nM for enterodiol and 0.55 nM for enterolactone were achieved. The within-run R.S.D. ranges from 3 to 6% and the between-run R.S.D. ranges from 10 to 14% for both enterolignans. This method allows simple, rapid, and sensitive quantification, and is suitable for measuring large numbers of samples.

Keywords: Lignans; Enterodiol; Enterolactone; Plasma; Liquid chromatography; Mass spectrometry

1. Introduction

Enterolactone and enterodiol (Fig. 1), also called enterolignans, are phytoestrogens with structural similarity to endogenous estrogens. Enterolignans have demonstrated antioxidant [1,2], and weak (anti-) estrogenic effects [3,4]. They are capable of induction of NADPH: quinone reductase (phase II enzymes) [5] and can inhibit enzymes involved in the metabolism of sex hormones (e.g. sex hormone binding globulin, 5α-reductase, and 17β-hydroxy-steroid dehydrogenase) [6–8]. Because of these activities, they may affect the development of several diseases. Epidemiological studies suggest that high serum concentrations of enterolactone are associated with a lower risk of acute coronary events [9,10]. Associations between enterolignans and cancer are unclear. Inverse associations for breast or prostate cancer were reported only in case-control studies [11–13], whereas no associations were found in three prospective studies [14–16] (reviewed by Arts and Hollman [17]).

Enterolignans are products of bacterial conversion of the plant lignans secoisolariciresinol, matairesinol, lariciresinol, and pinoresinol in the human colon [18]. Plant lignans are present in flax, grains, seeds, fruits and vegetables, olive oil, and beverages such as tea, coffee, and wine [19–23]. After consumption of these plant lignans, enterolactone and enterodiol are found as glucuronide and sulfate conjugates in human plasma, urine, and feces [24]. Due to different consumption patterns and variation in microflora, plasma concentrations of enterodiol and enterolactone vary widely between persons. For example, Kilkkinen et al. [25] reported enterolactone concentrations between 0 and 100 nM in men ($n = 1168$) and 0 and 180 nM in women ($n = 1212$); Adlercreutz et al. [24] found higher concentrations in vegetarians (up to 1000 nM; $n = 14$).

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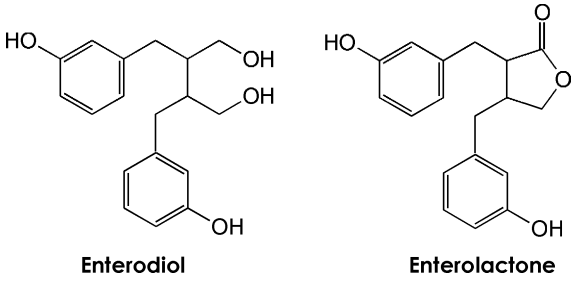

Fig. 1. Chemical structures of enterodiol and enterolactone.

Techniques that have been used to measure enterolignans are time-resolved fluorescence immunoassay, GC–MS, and LC in combination with UV, diode array, MS, or electrochemical detection. For routine measurements in human biological fluids, the immunoassay combines the advantage of high sensitivity with low costs. A major drawback of this method is that enterodiol, one of the two enterolignans, cannot be measured. Various aspects of method performance as well as benefits and limitations of the above mentioned techniques have been discussed by Wilkinson et al. [26].

Recently, Grace et al. [27] developed a LC–MS/MS method for isoflavones and lignans using $^{13}C_3$ labeled isotopes. A range of these phytoestrogens can be measured simultaneously with this method. We set out to adapt this method specifically for the quantification of enterodiol and enterolactone in human plasma. Our objective was to obtain a simple, straightforward and robust method applicable to the analysis of large numbers of samples, for instance from epidemiological studies. In this paper, we describe the results of the optimization of hydrolysis, extraction, and chromatographic conditions and evaluate the method's performance. The method described here is suitable for analyzing large numbers of samples due to a simple sample treatment, a short chromatographic run time, and simultaneous detection of both compounds.

2. Experimental

2.1. Materials

Pure standards of enterodiol and enterolactone were obtained from Fluka Chemie GmbH (Buchs, Switzerland). The internal standards, $^{13}C_3$-enterodiol and $^{13}C_3$-enterolactone (purity >97%; refers to the isotopic purity of the sum of all labeled C atoms, with C_0 <0.4%), were purchased form Dr. Botting (University of St Andrews, Scotland). β-Glucuronidase-sulfatase (EC 3.2.1.31) from *Helix Pomatia* (G7017, G1512) and β-glucuronidase from bovine liver (G0501) were obtained from Sigma (St. Louis, MO, USA). Another β-glucuronidase-sulfatase from *H. Pomatia* (cat. no. 104114) was obtained from Merck (Darmstadt, Germany). All other chemicals were of analytical grade and water was purified with a Milli-Q system.

2.2. Human test samples

Human plasma samples were obtained from two men and one woman aged 28 to 53 years. Plasma samples with relatively high concentrations of enterolignans (mean concentration of enterodiol: 7.0 nM; enterolactone: 39.2 nM), hereafter called 'high lignan plasma', were obtained after these subjects had consumed 25 g crushed flaxseed per day for 3 successive days. Plasma samples with low concentrations of enterolignans (mean concentration of enterodiol: 0.6 nM; enterolactone 5.4 nM), hereafter called 'low lignan plasma', were obtained after the same subjects had followed a diet poor in lignans for 3 successive days: they were not allowed to eat whole grain products, seeds, nuts and some specified fruits and vegetables. Plasma was prepared from venous blood samples drawn into vacuum tubes containing EDTA. The samples were centrifuged within 30 min at $1187 \times g$ for 10 min at 4 °C. Subsequently, the plasma samples of these three subjects were pooled and homogenized. Samples were stored at −80 °C until analysis.

2.3. Hydrolysis and extraction procedure

Total enterodiol and enterolactone concentrations were measured after hydrolysis of the conjugates using a freshly prepared enzyme mixture of β-glucuronidase-sulfatase from *H. Pomatia* (G1512) in sodium acetate buffer (0.5 M, pH 5.0). First, 10 μL of a mixture of $^{13}C_3$ labeled enterodiol and enterolactone (500 nM) was added into 4 mL vials. Subsequently, 300 μL plasma, 300 μL sodium acetate buffer (0.1 M, pH 5.0), and 60 μL enzyme mixture (2600 units β-glucuronidase) were added. The samples were incubated at 37 °C for 4 h and subsequently extracted twice with 1.5 mL diethyl ether. The samples were shaken with a Vortex mixer for 5 S and centrifuged after each extraction ($2300 \times g$, 10 °C, 10 min). The two ether fractions were combined and transferred into tubes containing 500 μL 40% methanol/water (v/v). The ether fraction was evaporated under a gentle stream of nitrogen at 30 °C with a Turbovap evaporator (Zymark, Hopkinton, MA, USA), after which the tubes were shaken with a Vortex mixer for 5 s. Prior to analysis, extracts were filtered through acrodiscs containing a 0.45 μm hydrophilic polyvinylidene fluoride membrane (Pall Corporation, Ann Arbor, MI, USA), transferred into vials, and injected into the LC–MS/MS system.

2.4. Chromatography and detection conditions

A Waters Alliance chromatography separation module 2690 (Milford, MA, USA) was used, which consisted of a chromatographic system equipped with a binary pump, and an auto sampler with a cooled sample tray kept at 10 °C. Separations were performed on an XTerra MS C_{18} column (50 mm × 3.0 mm, 5 μm; Waters, Milford, MA, USA), which was placed into a column oven set at 40 °C. The mobile phase

consisted of a mixture of water and methanol and was run at a flow rate of 0.4 mL/min. The gradient, starting at 10% methanol for 1 min, was increased linearly to 80% methanol in 6 min, which composition was kept for 0.5 min. Returning to the starting conditions in 0.5 min, the column was allowed to equilibrate in 3 min. The total run time was 11 min. The sample injection volume was 100 μL. The LC eluate was introduced into the mass spectrometer at 0.2 mL/min after a 50:50 (MS/waste) split. The divert valve was programmed to allow flow into the mass spectrometer from 4 to 9 min of each run.

2.5. Mass spectrometry

Detection was performed with a Micromass Quatro Ultima triple quadrupole mass spectrometer (Waters-Micromass, Manchester, UK) equipped with an electrospray probe. The mass spectrometer was operated in negative ion electrospray mode, with the capillary voltage at 2.5 kV. Nitrogen was used as desolvation gas and cone gas. Desolvation gas was used at a flow rate of 550 L/h and cone gas at a flow rate of 50 L/h. Source and desolvation gas temperatures were set at 120 and 350 °C, respectively. Dwell time was set at 0.5 s for each transition. Product ions were formed by collision-induced dissociation with argon as collision gas at a pressure of 2.3×10^{-3} m bar, and a collision energy ranging from 20 to 36 eV. Table 1 summarizes the characteristic precursor and product ions used for determination of enterodiol, enterolactone, and their internal standards. The most abundant fragment ion was used for quantification, while a second, less abundant, ion was used for confirmation by means of the observed ratio. In samples containing low concentrations of enterolignans confirmation was not always possible due to lack of sensitivity. Integration of peak areas was performed using MassLynx software (Waters-Micromass, Manchester, UK).

2.6. Calibration curves

Calibration curves were constructed in low lignan plasma (enterodiol 0.6 nM, enterolactone 5.4 nM). Calibration standards were freshly prepared for each series of analyses. Plasma samples were spiked with known concentrations of a standard mixture of enterodiol and enterolactone (0, 0.3, 1, 3, 10, 30, and 100 nM), and with 10 μL of a mixture of triply ^{13}C labeled enterodiol and enterolactone (500 nM). Subsequently, the plasma samples were hydrolyzed and extracted as described above. Calibration standards were injected at the start and at the end of each series of analyses. The average of the calibration standards within each series was used to obtain the calibration curve. Calibration curves were constructed by plotting the response factor (area enterolignan/area internal standard) against the concentration of the calibration standard. Because the plasma samples used for calibration contained small amounts of enterolignans, the response factor of a non-fortified plasma sample was subtracted from the response factor of the individual calibration samples. The calibration curves were forced through the origin.

Table 1
Precursor and product ion combinations of enterodiol, enterolactone, and their internal standards

Compound	Precursor ion	Product ion	Confirmation ion
Enterolignans			
Enterodiol	301.1	253.1	106.2
Enterolactone	297.1	253.1	107.2
Internal standards			
$^{13}C_3$-enterodiol	304.1	256.1	
$^{13}C_3$-enterolactone	300.1	255.1	

2.7. Optimization experiments

To find optimal conditions for hydrolysis and extraction of enterolignans in plasma, we used an HPLC method with electrochemical detection (coulometric), which had been developed to measure enterolignans in cell culture medium and intracellular fluids [28]. For quantification of both enterolignans, carbon working and reference electrodes were used, and the cell potential was increased to 650 mV. Four types of β-glucuronidase-sulfatases from *H. Pomatia*, and one type of β-glucuronidase from bovine liver were evaluated for the hydrolysis of the enterolignans. The amount of enzyme (range 120–3500 units), the incubation time (0–6.5 h), and temperature (37 and 50 °C) were varied in order to obtain complete hydrolysis. High lignan plasma was used in these experiments. When plasma samples with very low concentrations of enterolignans were analyzed with electrochemical detection, the resolution from interfering matrix components proved to be insufficient. To improve the resolution, we tested several columns: Chromolith (100 mm × 4.6 mm, Merck, Darmstadt, Germany), Symmetry (250 mm × 4.6 mm, 5 μm) and Nova-Pak (250 × 4.6 mm, 4 μm; 250 mm × 3.9 mm, 4 μm) from Waters (Dublin, Ireland), Discovery (250 mm × 4 mm, 5 μm, Supelco, Bellefonte, PA, USA), and Inertsil ODS 3 (250 mm × 4.6, 5 μm, Alltech, Deerfield, IL, USA). In addition, we varied the pH and type of buffer of the mobile phases (phosphate buffer pH 2.4, citrate buffer pH 3.7, and sodium acetate buffer pH 4.8–5.4) and tested several gradients (methanol, acetonitrile) for optimal separation of enterodiol and enterolactone from interfering peaks in plasma samples. Minor improvements in separation from matrix peaks were obtained. Therefore, HPLC with MS/MS detection, which has excellent specificity, was chosen instead as the preferred method.

2.8. Limits of detection

Limit of detection was determined by injection of 100 μL of five plasma samples with low amounts of enterodiol and enterolactone (range: 0.1–5.0 nM) on 10 different days. The limit of detection was defined as the amount of enterodiol or

enterolactone that resulted in a peak height three times the standard deviation of the baseline noise. The signal to noise ratio was calculated using MassLynx 4.0.

2.9. Recovery, within- and between-run variability

To calculate the recovery, low lignan plasma samples (enterodiol 0.6 nM, enterolactone 5.4 nM) were spiked with standard solutions of enterolignans (10, 30 and 100 nM). To assess the within-run variation, six identical high lignan plasma samples (concentration of enterodiol: 7.0 nM; enterolactone: 39.2 nM) were analyzed in one run. To assess the between-run variation, high and low lignan plasma samples were analyzed in duplicate on 22 separate days within a period of 3 months.

2.10. Stability of sample extracts

The stability at −80 °C of enterodiol and enterolactone in sample extracts was evaluated, to enable storage before analysis on LC–MS/MS. Ten different plasma samples were extracted and analyzed without and after storage at −80 °C for 9 days. The concentrations were compared using the paired Student's *t*-test for statistical analysis.

3. Results and discussion

3.1. Optimization of hydrolysis

Several enzymes with β-glucuronidase and/or sulfatase activity were tested in 300 μL high lignan plasma samples (enterodiol: 7.0 nM; enterolactone: 39.2 nM). β-Glucuronidase-sulfatase from *H. Pomatia* (G1512) had the highest response per unit of activity (data not shown). With this enzyme, we varied the amount of enzyme, and incubation time (Fig. 2). The highest yield was obtained with 1200 units β-glucuronidase between 2 and 6 h. In additional experiments the amount of enzyme was further increased (range 1300–3500 units) at 2–6 h. It showed that a 4 h incubation period at 37 °C was sufficient. The yield of both enterolignans increased approximately 20% when 2600 units enzyme were used for hydrolysis. When the amount of enzyme was increased further to 3500 units in a separate experiment the yield did not increase significantly (2600 units: enterodiol 31 ± 2.2 nM, enterolactone: 56 ± 2.0 nM; 3500 units: enterodiol: 31 ± 3.2 nM, enterolactone: 59 ± 3.3 nM). The final amount of enzyme (867 units/100 μL plasma) in our study is comparable with the amount of enzyme used by Grace et al. [27] (1205 units/100 μL serum). Other studies [29,30] reported lower amounts of enzyme (<150 units/100 μL plasma), or are not comparable because they used enzymes with differently defined activities. Most studies used an overnight incubation for the hydrolysis of enterolignans in plasma [27,29–33]. Of those studies, only Valentin-Blasini et al. [30] reported the optimization of hydrolysis. They showed that incubation with β-glucuronidase-sulfatase for 8 h is sufficient for complete deconjugation of daidzein. They also stated that the other analytes, including enterodiol and enterolactone, showed a similar deconjugation rate. In our study no differences were observed between incubation at 37 and 50 °C. Without enzyme, no enterolignan aglycones were detected in plasma (Fig. 2), which strongly suggests that all enterolignans in plasma are conjugated with glucuronic acid or sulfate. A similar observation has been reported by Smeds and Hakala [29], where in pooled unhydrolysed plasma only traces of enterolignans (below the quantification limit) were found.

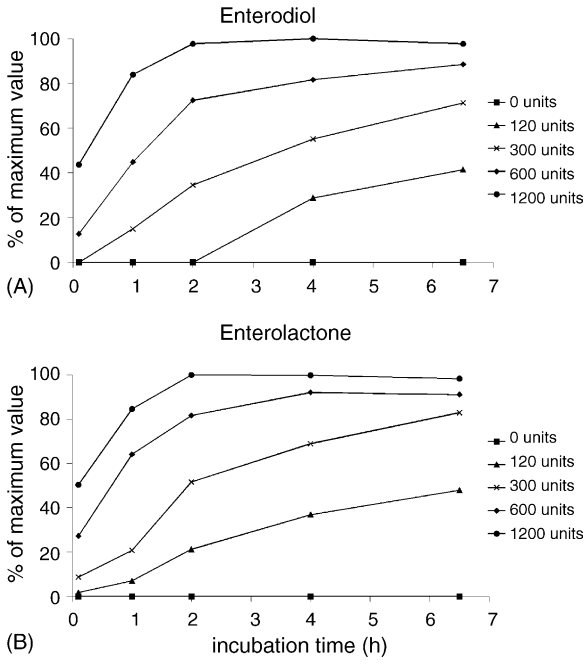

Fig. 2. Effect of incubation time and amount of β-glucuronidase-sulfatase from *Helix Pomatia* (G1512) on enterolactone (A) and enterodiol (B) release from plasma at 37 °C; values expressed as percentage of maximum value. The optimal hydrolysis conditions consisted of 4 h incubation with 2600 units enzyme/300 μL plasma.

3.2. Optimization of extraction

To optimize extraction conditions, plasma samples were spiked with known concentrations of a standard mixture of enterodiol and enterolactone, and the recovery was determined. Diethyl ether was used as an efficient and low-boiling extraction solvent. By varying the number of extractions and the volume of the diethyl ether, the optimal extraction conditions were determined. Extraction with 1.5 mL diethyl ether twice was as efficient as extraction with 1 mL diethyl ether three times (data not shown). After extraction, diethyl ether has to be removed. However, when the diethyl ether fraction was evaporated to dryness and the residue re-dissolved in 50% methanol/sodium acetate buffer (0.1 M, pH 5.0) (v/v), more than 75% of the enterolignans was lost. To prevent this loss, the ether fractions were

transferred to tubes containing a solvent capable of readily dissolving enterolignans, and immiscible with ether. In this way, the lignans were kept in solution. When sodium acetate buffer (0.1 M, pH 5.0) was used alone as immiscible solvent, the recovery of enterodiol and enterolactone was 54 and 15%, respectively. Several other solvents were tested to improve the recovery. The highest recovery (88% for enterodiol, and 104% for enterolactone) was obtained with a mixture of 50% methanol/sodium acetate buffer (0.1 M; pH 5.0; v/v). A mixture of 50% acetonitrile/sodium acetate buffer (v/v) was not appropriate because it produced heavily tailing peaks in the chromatograms.

3.3. Optimization of chromatography and detection

Several column types and chromatographic conditions were tested in order to develop a short, though robust and sensitive analytical method. A short (50 mm × 3.0 mm) Xterra column run with a methanol/water gradient was selected, providing the best compromise between selectivity and speed of analysis. The overall analysis time was only 11 min. The use of ammonium acetate buffer, described in the method of Grace et al. [27], was abandoned as this led to a reduced sensitivity of the enterolignans by mass spectrometric detection. Mass spectrometric conditions were optimized for the detection of two product ions for both enterolignans and of one product ion for the $^{13}C_3$ labelled analogs. The most abundant product ion was used for quantification and the second product ion for confirmation of the identity of the analyte. In this way the risk of 'over'-quantification due to co-eluting matrix components could be greatly reduced. In low lignan containing plasma samples, however, confirmation was not always possible.

The solvent composition of the injected extracts was found to be a critical parameter. The injection of samples containing 50% methanol/water (v/v) resulted in asymmetric and poor peak shapes. This was due to the fact that the sample is injected in mobile phase containing only 10% methanol. It was decided to reduce the methanol content of the sample. The peak shapes improved when the methanol content in the sample was reduced to 40%. This had little effect on the observed recovery of the enterolignans. Furthermore, because we used internal standards for enterodiol and enterolactone, possible losses during extraction and re-dissolution steps were corrected for automatically. Representative chromatograms of enterodiol and enterolactone with their internal standards are shown in Fig. 3.

We compared the slopes of calibration curves constructed 40% methanol/water (v/v) ($n = 5$) with calibration curves constructed in plasma ($n = 5$), analyzed within the same run. For enterodiol calibration curves constructed in 40% methanol/water (v/v) had 0–30% (mean 15%) higher response factors (area enterolignan/area internal standard) than calibration curves constructed in plasma, and for enterolactone calibration curves constructed in 40% methanol/water (v/v) had 0–15% (mean 10%) higher response factors. Although triply ^{13}C labeled internal standards were used, we observed that plasma matrix ion suppression was not sufficiently corrected for when using the calibration curve in methanol/water. Therefore, standard curves in blank human plasma are preferred. Valentin-Blasini et al. [30] observed no significant matrix effect of serum on

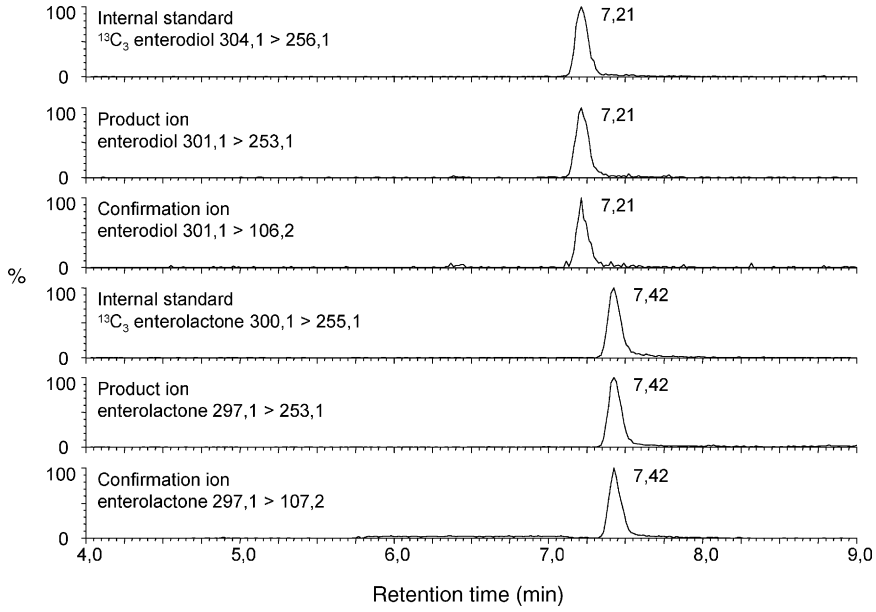

Fig. 3. Chromatograms (unsmoothed) of enterodiol, enterolactone, and their internal standards in a quality control sample (high lignan plasma). The chromatograms show the MRM transitions that were used for quantification and confirmation. From bottom to top: internal standard $^{13}C_3$ enterodiol (304.1 > 256.1), product ion enterodiol (301.1 > 253.1), confirmation ion enterodiol (301.1 > 106.2), internal standard $^{13}C_3$ enterolactone (300.1 > 255.1), product ion enterolactone (297.1 > 253.1), and confirmation ion enterolactone (297.1 > 107.2).

Table 2
Detection limits, within- and between-run variation for enterolignans in plasma

	n	Enterodiol	Enterolactone
Limit of detection (S/N = 3; nM)[a]	10	0.15 ± 0.1	0.55 ± 0.4
Within-run R.S.D. (%)	6	5.8	3.2
Between-run R.S.D. (%) high[b]	22	14	10
Between-run R.S.D. (%) low[c]	22	21	13

[a] Mean ± S.D.
[b] High lignan plasma (enterodiol: 7.0 nM; enterolactone: 39.2 nM).
[c] Low lignan plasma (enterodiol: 0.6 nM; enterolactone: 5.4 nM).

calibration curves. However, they used fetal bovine serum as matrix, whereas we used a homogenized mixture of plasma from three humans. Furthermore, their analytical procedure differed from ours. This might explain the lack of matrix effect. The calibration curves for enterodiol and enterolactone were found to be linear over the concentration range used, with correlation coefficients ≥0.99. The slope of the calibration curve of enterodiol was 0.237 ± 0.042 and that of enterolactone 0.096 ± 0.015. The limit of detection determined in plasma samples was 0.15 nM for enterodiol and 0.55 nM for enterolactone (Table 2). Comparable or higher detection limits have been reported for time-resolved fluorescence immunoassay detection of enterolactone in plasma (0.35 nM) [32], for GC with MS detection of enterodiol and enterolactone in plasma (0.2–1.0 nM) [34], for LC with MS detection of enterodiol and enterolactone in plasma or serum (0.4–0.5 nM [27], 0.3–3.6 nM [30]), and for LC with electrochemical detection (1.9–2.1 nM) [33]. Smeds and Hakala [29] and Grace et al. [27] reported much lower detection limits for LC–MS (<30 pM), but these detection limits were determined in the absence of plasma.

3.4. Recovery, within- and between-run variation

With each series of analyses, quality control samples were included. The recovery, and within- and between-run variation of the method were assessed with these control samples, which contained high (enterodiol: 7.0 nM; enterolactone: 39.2 nM) and low (enterodiol: 0.6 nM; enterolactone 5.4 nM) concentrations of enterolignans. Recovery of enterolignans added prior to the hydrolysis procedure varied from 97 to 107% (Table 3). These data are in accordance with recoveries reported elsewhere [24,27,30,32]. Lower recoveries (68–85%) of enterolignans in plasma were reported using LC with electrochemical detection [33]. The within-run variation of our method was quite satisfactory. The R.S.D., at a concentration of 7.0 nM for enterodiol and 39.2 nM for enterolactone, ranged from 3 to 6% (Table 2). Comparable R.S.D.s are reported by other authors [27,35]. Nurmi et al. [33] reported a high within-run variation for enterodiol (42%) but similar variation for enterolactone (1.5%). Slightly higher variations (9–20%) were reported by Smeds and Hakala [29]. The between-run variation of our method was relatively high; the R.S.D. in high enterolignan plasma (enterodiol: 7.0 nM; enterolactone 39.2 nM) ranged from 10 to 14% for both enterolignans, and from 13 to 21% in low enterolignan plasma (enterodiol: 0.6 nM; enterolactone 5.4 nM). Lower between-run variations (3.7% for enterodiol; 3.3% for enterolactone) were reported by Grace et al. [27] using LC–MS/MS at concentrations of >30 nM, and by Valentin-Blasini et al. [30] (5.1% for enterodiol; 4.5% for enterolactone) at concentrations of >15 nM. Both authors constructed their calibration curves in the absence of plasma. When calibration curves of enterodiol and enterolactone are constructed in plasma the R.S.D.s of the slopes are around two-fold higher than in the absence of plasma [29]. The use of calibration curves constructed in plasma might explain the relatively high R.S.D.s of the quality control samples of our method.

Table 3
Recovery of enterolignans in plasma

Spike (nM)	*n*	Recovery (%)[a]	
		Enterodiol	Enterolactone
10	13	99 ± 11	103 ± 20
30	13	107 ± 7	107 ± 11
100	10	97 ± 4	99 ± 2

[a] Mean ± S.D.

3.5. Stability of sample extracts

When sample extracts could not be analyzed immediately, they were stored at −80 °C. Stability tests indicated that after 9 days of storage at −80 °C the enterodiol concentration in plasma decreased by 3 ± 10% (n = 10), whereas that of enterolactone did not change 0 ± 6% (n = 10). The decrease in enterodiol was not statistically significant (paired Student's t-test; p = 0.95 for enterodiol). Therefore, it was concluded that sample extracts can be stored for 9 days at −80 °C without affecting the concentration of both enterolignans.

3.6. Levels of enterolignans in plasma samples

The LC–MS/MS method described in this paper was successfully applied to quantify enterolignans in plasma after supplementation of purified secoisolariciresinol diglucoside to healthy men and women [36]. Furthermore, this method is currently being used to quantify concentrations of enterolignans in epidemiological studies. Preliminary results showed that the median plasma concentration for enterolactone was 9.2 nM, and for enterodiol 1.0 nM (n = 807). The range of enterolignan concentrations was very wide. The three highest concentrations observed for enterolactone were 687, 564, and 298 nM, and for enterodiol 184, 86, and 78 nM. The distribution of plasma enterolignans appeared skewed to higher values. The lowest concentrations observed were below the detection limits for both compounds.

4. Conclusions

We developed and validated a LC–MS/MS method using $^{13}C_3$ labeled isotopes for the simultaneous quantification of total enterodiol and enterolactone concentrations in plasma. This method allows detection and quantification of nanomolar concentrations of these enterolignans. By optimizing the hydrolysis and extraction specifically for enterodiol and enterolactone, an efficient and adequate method was developed. Our method proved itself to be useful for the analyses of large numbers of plasma samples in a wide range of concentrations and is currently used for the analysis of enterolignans in plasma samples collected from large epidemiological studies.

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