Journal of Chromatography, 617 (1993) 173–179
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6904

Quantification of mammalian lignans in biological fluids using gas chromatography with ion mobility detection

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(First received February 9th, 1993; revised manuscript received May 5th, 1993)

ABSTRACT

A method is presented to quantify selected mammalian lignans in human physiological fluids by gas chromatography (GC) coupled with ion mobility spectrometry (IMS). The use of IMS following GC permitted the selective and sensitive measurement of 2,3-bis(3-hydroxybenzyl)butane-1,4-diol (*i.e.*, enterodiol) and *trans*-2,3-bis(3-hydroxybenzyl)-γ-butyrolactone (*i.e.*, enterolactone) concentrations in urine and plasma following dietary supplementation with whole wheat/flaxseed bread high in mammalian lignan precursors. Following six weeks of flaxseed feeding, urinary and plasma levels of enterodiol and enterolactone were elevated, exceeding the amounts found at baseline by a factor of 3–5. The approach to mammalian lignan methodology presented herein provides novel analytical phytochemical procedures for assessing the impact of lignan consumption in human health and disease.

INTRODUCTION

Specific naturally occurring dietary constituents have been shown to have an inhibitory effect on carcinogenesis [1–3]. Two of these compounds, 2,3-bis(3-hydroxybenzyl)butane-1,4-diol and *trans*-2,3-bis(3-hydroxybenzyl)-γ-butyrolactone, known respectively as enterodiol and enterolactone, are the major mammalian lignans present in the urine and serum of humans and animals [2–7]. Certain fiber-rich foods, such as flaxseed, contain plant lignans which serve as precursors for the synthesis of enterodiol and enterolactone [2,3,7–12]. Dietary and cancer intervention studies will benefit greatly from the development of precise and accurate analytical methods for quantifying mammalian lignans in biological fluids.

Very little is known about the levels of lignans present in human blood. Enterolactone has been the only lignan to be quantified in human blood [6,10]. Enterolactone levels were determined using gas chromatography (GC)–isotope dilution mass spectrometry (MS) [6,10]; attempts to determine enterodiol concentrations were considered unsuccessful [10]. This study reports both enterodiol and enterolactone concentrations in human plasma and urine, before and after whole wheat/flaxseed supplemented dietary intervention. A novel approach to mammalian lignan quantification is presented here involving the application of GC coupled with ion mobility spectrometry (IMS) detection. IMS is a gas phase, atmospheric pressure detection technique in which ions are propelled by an electric field through a counter-

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0378-4347/93/$06.00

current gas flow [13–16]. The ions are separated based on their charge, mass, and collisional cross-sectional area and can be distinguished by their arrival time at a collector electrode [17,18].

IMS is inherently a low-resolution technique. Therefore, complex samples such as biological fluids require chromatographic separation before IMS detection. The use of GC preceding IMS reduces analyte interferences and avoids detector overload and matrix effects. GC followed by IMS has also been used for complex sample matrices such as gasoline, plant extracts, and the analysis of opiates, barbituates, and other drugs in urine [19–21]. The tunable selectivity of IMS allows the elimination of interfering chromatographic peaks, assuming that near-simultaneous eluting compounds have different ion mobilities.

EXPERIMENTAL

Dietary procedures

As stated in the introduction, the main goal of this work was to quantify mammalian lignans in both urine and plasma in conjunction with a feeding study designed to increase levels of these compounds in humans. The feeding study was conducted to determine how short-term consumption of a lignan-containing flaxseed-fortified bread would affect lignan profiles in urine and plasma of adult male subjects [28]. The primary experimental group was comprised of six men. A whole wheat/flaxseed (WWF) bread high in lignan precursors was fed to them for six consecutive weeks. Otherwise, each subject consumed his usual diet. A daily supplement of six slices of WWF bread (Flax N' Honey, Natural Ovens of Manitowoc, WI, USA) provided 13.5 g of stabilized human-grade flaxseed flour per day. Each subject was instructed to follow his normal dietary patterns except to substitute WWF bread for high-fiber grain foods, thereby avoiding other foods high in mammalian lignan precursors [2,3,8]. The WWF bread was labelled and distributed weekly to each subject. The protocol was approved by the Washington State University Committee on Human Studies. For purposes of analytical procedures development, biological samples from one healthy Caucasian man, age 34 years, was selected for study.

Sampling procedures

Urine collections (24 h) were obtained at time 0 (pre-dietary intervention baseline) and following six weeks of dietary intervention. These samples were collected in plastic bottles containing ascorbic acid (1 g/l) as an antioxidant. Urine volumes were measured and four 10-ml aliquots were stored frozen at −40°C. Creatinine content verified the completeness of collection [22].

Fasting (12 h) 30-ml blood samples were obtained between 7 and 8 a.m. from the antecubital vein of each subject at time 0 and week 6. Blood samples were drawn into 10-ml heparinized Vacutainer tubes. The plasma was divided into 1-ml aliquots and frozen at −40°C.

Preparatory analytical procedures

Urinary lignans. Enterodiol and enterolactone were extracted, derivatized, and chromatographed as previously described with slight modifications [7,23]. Initially, the frozen aliquots of urine (10 ml) were thawed and pH adjusted to 4.5 with 3 *M* acetate buffer and [6,9-^{3}H]estriol-16α-(β-D-glucuronide) (20 Ci/mmol; Amersham, Arlington Heights, Il, USA) added as an internal standard. The urine was centrifuged to remove solids and passed through a preconditioned cartridge of reversed-phase octadecylsilane-bonded silica (Sep-Pak C_{18}, Waters, Milford, MA, USA) to extract the lignans. Following application of the urine to the C_{18} column, the column was washed with 10 ml of water and the free and conjugated compounds (neutral and phenolic steroids and other phenolics) eluted with 4 ml of methanol. Urinary lignans exist primarily as glucuronidated conjugates (*i.e.*, 92% of enterodiol and 98% of enterolactone [24]). The methanol fraction of the C_{18} Sep-Pak extraction was loaded on a QAE-Sephadex A-25 column (Pharmacia LKB Biotechnology, Piscataway, NJ, USA) in the acetate form (2.5 cm × 0.5 cm I.D.). The column was washed with 4 ml of methanol and 15 ml of 0.15 *M* formic acid in methanol, and the lignan conjugates eluted and collected with 15 ml

of 0.60 *M* formic acid in methanol. The lignan-containing fraction was evaporated to dryness under nitrogen at 45–50°C. Subsequent hydrolysis (16–18 h) of the conjugated fraction with 9000 U of β-glucuronidase (Type H-1, Sigma, St. Louis, MO, USA) in 0.1 *M* acetate buffer, pH 4.6, containing 30 m*M* ascorbic acid yielded free lignans. Free lignans were then concentrated on a C_{18} Sep-Pak column and eluted with 4 ml of methanol. The methanol fraction was dried under nitrogen at 45–50°C and redissolved in an aqueous sodium hydroxide, sodium bicarbonate, and ascorbate solution. The free lignans were then extracted from the aqueous solution with 5 ml of ethyl acetate and washed twice with 0.5 ml of water. The ethyl acetate fraction was dried under nitrogen, derivatized to form trimethylsilyl (TMS) esters and extracted with 0.5 ml hexane.

Plasma lignans. Lignans were extracted and purified by selecting and applying previously described methodologies for plasma steroid hormones and lignans [7,23,25,26]. Frozen aliquots of plasma (1 ml) were thawed and the pH adjusted to 5 with 1.5 *M* acetate buffer. [6,9-^{3}H]Estriol-16α-(β-D-glucuronide) was added as an internal standard. Water was added to give a 10-ml final volume, followed by the addition of 0.5 *M* triethylammonium sulfate (TEAS) and 20 n*M* norgestrel. Consequently, when estrogenic-like compounds (*i.e.* glucuronidated lignans) are extracted from plasma, the steroid–protein interactions will be counteracted by the presence of TEAS and norgestrel at pH 5, resulting in quantitative recoveries of glucuronide-bound steroids [27]. After standing for 30 min at room temperature, the sample was applied to a preconditioned C_{18} reversed-phase extraction column. Following application of plasma to the C_{18} column, the column was washed with 10 ml of 0.15 *M* acetate buffer (pH 5) and free and conjugated compounds eluted with 4 ml of methanol. Plasma enterodiol and enterolactone were derivatized as previously described for urinary lignans.

Standards. [6,9-^{3}H]Estriol-16α-(β-D-glucuronide) was used as an internal standard for recovery during extraction, hydrolysis, and purification of the samples up to the GC–IMS step, since it behaves identically to the endogenous lignan glucuronides throughout the procedure [10,23,27]. This does not necessarily imply that the conditions are optimal for the hydrolysis of lignan glucuronides; however, in the absence of authentic radioactive lignan glucuronide conjugates it appears to offer an acceptable substitute [10,23,27]. The recovery of radiolabeled estriol derived from [6,9-^{3}H]estriol-16α-(β-D-glucuronide) added to urine and plasma ranged from 74 to 80%. All assays were performed in triplicate. Urinary fasting and WWF feeding enterodiol inter-assay variabilities were 5% (n = 3) and 11% (n = 3), respectively, while fasting and WWF feeding enterolactone variabilities were 6% (n = 3) and 9% (n = 3), respectively. Plasma fasting and WWF feeding enterodiol inter-assay variabilities were 9% (n = 3) and 30% (n = 3), respectively, while fasting and WWF feeding enterolactone variabilities were 6% (n = 3) and 5% (n = 3), respectively.

Purified mammalian lignans (enterodiol and enterolactone) were purchased from Dr. Tapio Hase, Department of Chemistry, University of Helsinki (Helsinki, Finland). Purity of the aforementioned mammalian lignan standards and their TMS derivatives were verified in our laboratory by high-performance liquid chromatography–thermospray MS [28]. The fragment pattern correlated closely with data reported from GC–electron impact MS [5].

Instrumental procedures

Analysis was conducted using a Hewlett-Packard 5890 gas chromatograph (Hewlett-Packard, Avondale, PA, USA). Chromatograms were collected using a Hewlett-Packard Model 3392A integrator. The chromatographic column was a 27 m × 0.25 mm I.D. capillary, with a 5% phenylmethyl silicone stationary phase (DB-5) and a 1-μm film thickness (J&W Scientific, Folsom, CA, USA). The chromatographic temperature program used for all runs was: initial temperature 150°C for 1 min, temperature ramp at 7.5°C/min up to 275°C and hold for 10 min. The injector temperature was set at 175°C for all runs, since standards appeared to degrade in the injector at higher temperatures.

The in-house built ion mobility spectrometer was coupled to the gas chromatograph using a capillary direct interface. The ion mobility spectrometer was run at 225°C with a drift gas flow-rate of 1400 ml/min. Prepure nitrogen was used as the drift gas. The ion mobility drift field was held at 260 V/cm and the gate closure voltages were 60 V. Drift times were determined with standards using the Fourier transform mode of the ion mobility spectrometer and chromatograms were taken using the monitor mode, which allows the measurement of the ion current summed over a tunable range of mobility values. Chromatographic retention times of the derivatized lignan standards were verified using GC–MS. The urine and plasma samples were run initially with the ion mobility spectrometer in the non-selective mode (monitored from 6 to 20 ms) to verify retention times and pinpoint possible interfering peaks. Since the derivatized lignans have nearly identical drift times, the detector was set to monitor the drift times (12.2–15.8 ms) of both lignans simultaneously.

Urine enterodiol and enterolactone concentrations were determined using a calibration curve developed from standards. Data were adjusted for urine volume dilution and lignan recoveries as determined in the preparatory analytical procedures.

Plasma enterodiol and enterolactone concentrations were determined using the method of standard additions. To each of four 100-μl aliquots of each derivatized plasma extract was added 100 μl of one of the calibration standards. Hexane (100 μl) was added to a fifth aliquot of each derivatized plasma extract as a blank addition; hexane was the solvent used for all solutions. The plasma extract concentrations were determined from the x-intercept derived using a linear regression algorithm on the addition curves. The raw data were adjusted for the plasma volume dilution and lignan recovery, as determined in the extraction procedure.

RESULTS AND DISCUSSION

Although the primary experimental group was comprised of six men, only one randomly chosen participant's biological samples were chosen for the development of the analytical procedures. Fig. 1 shows the ion mobility spectra of TMS-derivatized enterodiol and enterolactone standards. These spectra were used to determine which drift times to monitor for selective ion detection. The window of 12.2–15.8 ms was chosen so both lignans could be detected simultaneously with a high degree of selectivity from other compounds in the sample. Figs. 2 and 3 show the effect of non-selective and selective mobility monitoring on derivatized urine and plasma extract chromatograms, respectively. TMS-derivatized standards and samples containing 200–800 pg of enterodiol and enterolactone were injected on-column to acquire the data presented in Figs. 1–3.

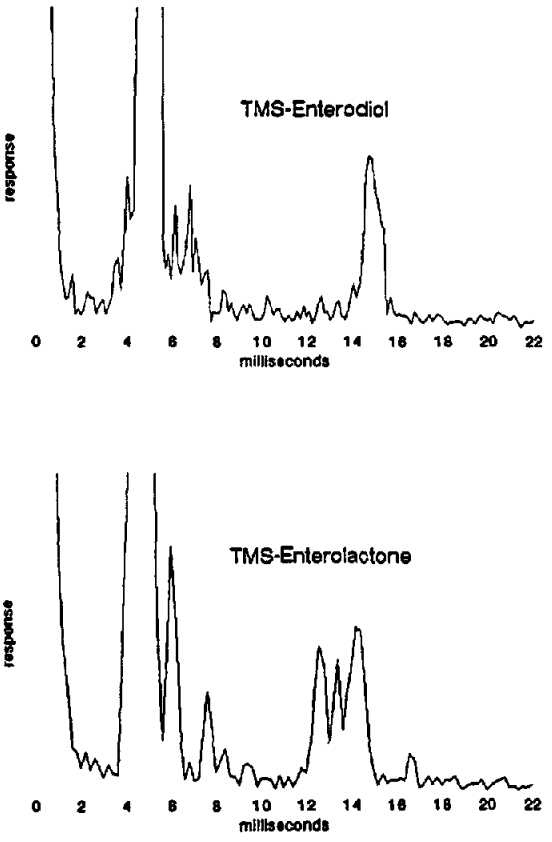

Fig. 1. Ion mobility spectra of TMS-derivatized enterodiol and enterolactone standards. Fourier transform positive mode with air drift gas. Frequency scan range: 20–15 020 Hz; scan time: 4.0 s.

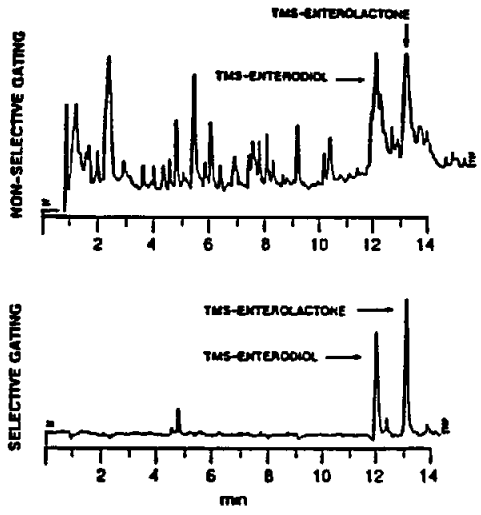

Fig. 2. Effect of selective gating on urinary extract chromatograms. Non-selective gating monitored 6–20 ms and selective gating monitored 12.2–15.8 ms.

The enterodiol and enterolactone calibration curves are shown in Fig. 4. The on-column quantities were calculated using standard concentrations, injector split ratio, and injection volume. The response was the baseline-to-baseline integration value computed by the Hewlett-Packard integrator. Selective ion monitoring mode (12.2–15.8 ms) was used for all runs. Regression analysis of the calibration curve data resulted in a good linear fit for both enterodiol and enterolactone, with r^2 values of 0.957 and 0.987, respectively.

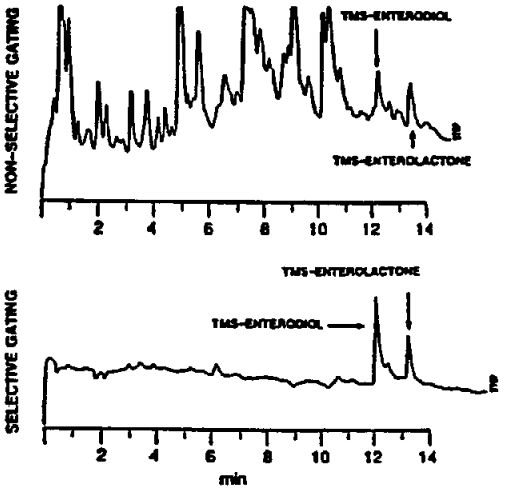

Fig. 3. Effect of selective gating on plasma extract chromatograms. Non-selective gating monitored 6–20 ms and selective gating monitored 12.2–15.8 ms.

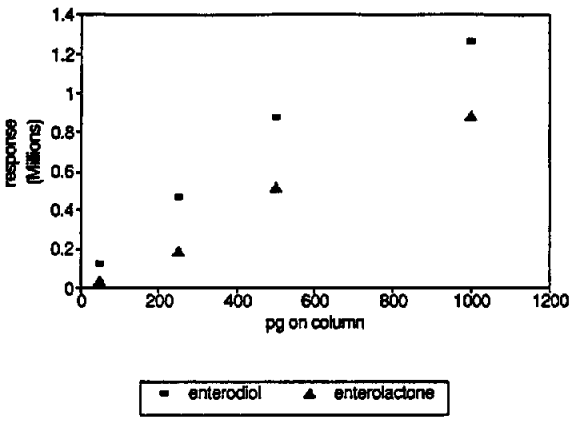

Fig. 4. Lignan standard calibration curve.

Enterodiol and enterolactone 24-h excretion amounts for urine are given in Table I. On-column quantities for the derivatized extracts were developed from the regression equation of the calibration curve, injector split ratio, and injection amount. The on-column amounts were correlated to the original urine sample by adjusting for the volume dilution following extraction and the extraction recoveries. Enterodiol and enterolactone concentrations for plasma are presented in Table II. The method of standard additions was used for plasma enterodiol and enterolac-

TABLE I

URINE CONCENTRATIONS FROM ONE SUBJECT

Lignan	Amount excreted (mean ± S.D.[a]) (μmol/24 h)
Urine collected before WWF feeding	
Enterodiol	1.57 ± 0.08
Enterolactone	6.48 ± 0.38
Urine collected after six weeks WWF feeding	
Enterodiol	4.29 ± 0.47
Enterolactone	30.65 ± 2.9

[a] Means and standard deviations were derived from triplicate assays of a single sample.

TABLE II

PLASMA CONCENTRATIONS FROM ONE SUBJECT

Lignan	Plasma concentration (mean ± S.D.[a]) (ng/ml)
Plasma collected before WWF feeding	
Enterodiol	13.9 ± 1.3
Enterolactone	29.0 ± 1.8
Plasma collected after six weeks WWF feeding	
Enterodiol	45.9 ± 14
Enterolactone	147 ± 7.2

[a] Means and standard deviations were derived from triplicate assays of a single sample.

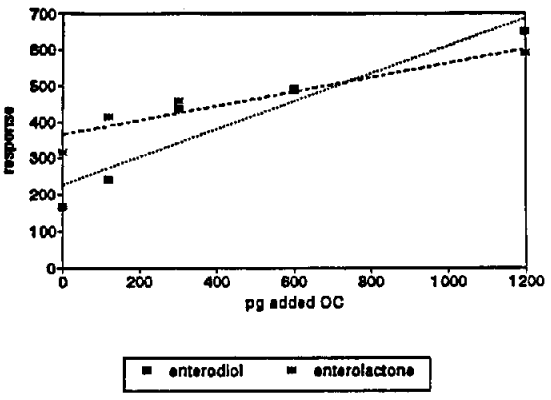

Fig. 6. Standard addition curve for plasma enterodiol and enterolactone after six weeks WWF feeding.

tone concentration determinations. The addition curve for the initial plasma extract is shown in Fig. 5. The addition curve for plasma after the six-week feeding study is shown in Fig. 6.

The values obtained for enterodiol and enterolactone concentrations in urine are similar to previous reports [10,27,28]. Increased urinary excretion of these lignans after dietary intervention also correlate with previously reported values [28]. Plasma baseline enterolactone concentrations found in this study are similar to the range of values reported for men [6], while WWF feeding resulted in elevated enterodiol and enterolactone concentrations. However, baseline plasma enterolactone concentrations reported here are over a factor of 10 higher than those previously reported for women [10]. This discrepancy in quantitative values may be accounted for by the difference in extraction column resin, as some extraction column stationary phases have been shown to destroy lignans [23]. Plasma baseline enterodiol and dietary intervention enterodiol and enterolactone concentrations have not been previously reported.

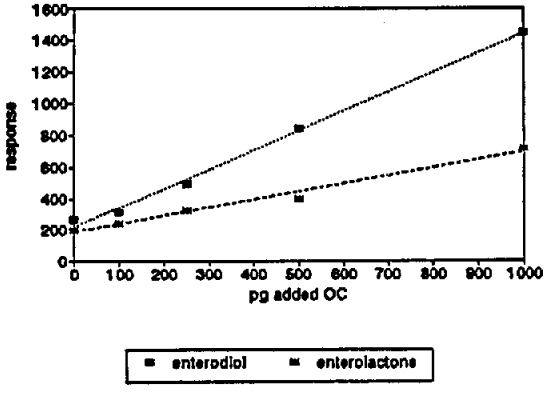

Fig. 5. Standard addition curve for plasma enterodiol and enterolactone before WWF feeding.

Axelson *et al.* [8] determined that plant lignan precursors of mammalian lignans were found in increasing order of amount in barley, oat, soya, buckwheat, rye, and flaxseed. These investigators reported that the addition of flaxseed (10 g per day) to two male subjects' diets resulted in five- to ten-fold increases in urinary enterodiol and enterolactone over a five-day feeding period. This study further extends and supports these observations by showing increases in the concentrations of urinary and plasma enterodiol and enterolactone following six weeks of flaxseed feeding. Enterolactone has been found to be present in the highest concentration in urine, followed by enterodiol [2,3,23,27]. This finding is consistent with results of this study where urinary and plasma enterolactone concentrations were found to be two- to seven-fold greater than enterodiol.

A recent review [29] reported the potential protective roles of lignans in the etiology of breast, endometrial, prostate, and colon cancers, implying that lignans may play a role as anticancer

agents. Further research is warranted to investigate the development of precise and accurate analytical techniques capable of quantifying specific plant and mammalian lignans possibly related to reduction in cancer incidence through dietary intervention.

CONCLUSIONS

The use of GC–IMS allows a sensitive and selective means of determining amounts of derivatized lignans in biological fluids. The unique IMS product ion spectra of the lignans allow a high degree of selectivity which is essential when dealing with extremely complex matrices such as urine and plasma. IMS sensitivity and selectivity facilitates the measurement of enterodiol levels in plasma, which have not been previously reported. Results of the dietary intervention show that a WWF-supplemented diet induces a significant increase in lignan levels in both urine and plasma.

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