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Urinary excretion of lignans after administration of isolated plant lignans to rats: the effect of single dose and ten-day exposures

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Abstract

The difference in urinary excretion of mammalian and plant lignans in rats was determined after oral administration of equivalent doses (25 mg/kg of body weight) of 7-hydroxymatairesinol (HMR), lariciresinol (LAR), matairesinol (MR), and secoisolariciresinol (SECO). Twenty-four hours-urine samples were collected after a single dose and after administration of one dose/day for 10 days. Eight lignans were analysed in urine extracts using a high-performance liquid chromatography–tandem mass spectrometry method showing good sensitivity and repeatability. After a single dose of HMR, LAR, MR, and SECO, the main metabolites were 7-hydroxyenterolactone (HENL), cyclolariciresinol (CLAR), enterolactone (ENL), and enterodiol (END), respectively, but after 10-day exposure ENL was the main metabolite of all the tested lignans, showing a considerably higher excretion than after a single dose. Metabolic transformations of plant lignans into each other could also be observed.

Keywords: 7-Hydroxymatairesinol; Matairesinol; Secoisolariciresinol; Lariciresinol; Cyclolariciresinol; Enterolactone; 7-Hydroxyenterolactone; Enterodiol; Plant lignans; Mammalian lignans; HPLC–MS/MS

1. Introduction

Plant lignans such as matairesinol (MR), 7-hydroxymatairesinol (HMR), and secoisolariciresinol (SECO) both as an aglycon and as a diglycoside (SDG) are converted to the mammalian lignans enterodiol (END) and/or enterolactone (ENL) (Fig. 1) both in vivo [1–6] and in vitro using fecal microbiota [7,8]. HMR has been found to convert also to 7-hydroxyenterolactone (HENL) (Fig. 1) both in vivo (in rats) [3] and in vitro using human fecal microbiota [7]. High concentrations of ENL in serum and urine are suggested to be associated with a lower breast cancer risk in women [9–11] and with a reduced risk of coronary heart disease [12,13]. ENL, as well as its precursors SDG and HMR, has also shown anticarcinogenic properties in dimethylbenz[*a*]-anthracene induced experimental mammary carcinoma in rats [3,5,14,15]. Because of these putative favourable health effects, a high ENL concentration in the body may be desirable. Administration of ENL as such was shown to be superior to plant lignans in increasing the urinary ENL excretion in rats (within 24 h after a single dose of each lignan) [2].

Plant lignans are present in our diet; SECO and MR are found in e.g., legumes, nuts, grains, berries, fruits, and espe-

Abbreviations: bw, body weight; CLAR, cyclolariciresinol; END, enterodiol; ENL, enterolactone; HAc, acetic acid; HENL, 7-hydroxyenterolactone; HMR, 7-hydroxymatairesinol; HPLC–MS/MS, high-performance liquid chromatography–tandem mass spectrometry; IC, internal control; LAR, lariciresinol; LOD, limit of detection; MeOH, methanol; MR, matairesinol; MRM, multiple reaction monitoring; MUG, 4-methylumbelliferyl-β-D-glucuronide; MUS, 4-methylumbelliferyl sulphate; QC, quality control; R.S.D., relative standard deviation; SDG, secoisolariciresinol diglycoside; SECO, secoisolariciresinol

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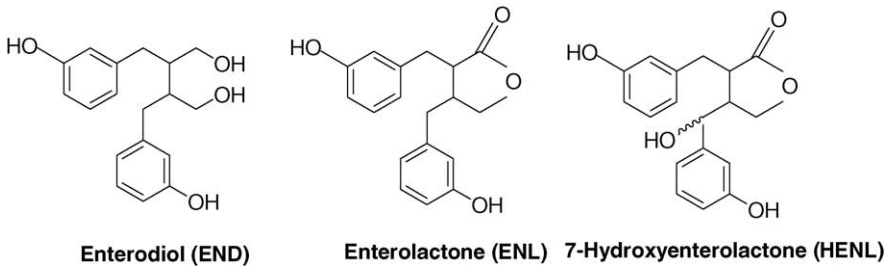

Fig. 1. Enterolignan metabolites of plant lignans.

cially in flaxseed [16]. The plant lignan lariciresinol (LAR) identified in e.g., rye bran was shown to be a noteworthy precursor of END and ENL in an in vitro study [7]. Several lignans are also present in wood species. HMR is very abundant in knots of Norway spruce (*Picea abies*) and also e.g., MR, SECO, and LAR are present in wood knots [17]. These lignans, especially HMR, can easily be isolated from wood knots in large scale. Moreover, we have recently shown that HMR can easily be transformed to other lignans such as (+)-LAR, (+)-cyclolariciresinol (CLAR), 7-oxomataresinol, (−)-MR, (−)-END, and (−)-ENL by chemical modification [18–20].

In the search of precursors forming ENL we have studied the metabolism in rats after oral administration of several potential mammalian lignan precursors. In a previous study we showed that within 24 h after administration of a single oral dose (25 mg/kg of body weight (bw)) of plant lignans to rats, the order of urinary ENL excretion was MR > SECO ≥ SDG ≥ HMR [2]. In the present study, we included LAR isolated from wood knots and extended the experiment to 10 days in order to study the effect of prolonged exposure on the formation of mammalian lignans. The study gives important information as very few comparative studies on lignan metabolism with pure compounds have been done before in vivo.

The urinary excretion of plant and mammalian lignans was measured after acute (single dose) or sub-chronic (10 days) exposure to the plant lignans HMR, LAR, MR or SECO. A high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) method using electrospray ionisation for quantitation of the lignans in rat urine extracts was developed by modification of our previous method [21].

2. Experimental

2.1. Chemicals

(−)-HMR, (−)-SECO, and (+)-LAR were extracted from coniferous trees; HMR and SECO as described previously [2]. LAR was isolated from ground knots of *Pinus cembra* by Soxhlet extraction with hexane to remove the lipophilic extractives and then with acetone to obtain the hydrophilic extractives including LAR. The acetone extract was purified using flash chromatography. The purity of the final product was 97% as determined by GC. HMR was a mixture of two stereoisomers differing in stereochemistry at C-7, HMR1 [(−)-allo-HMR] and HMR2 [(−)-HMR] with a HMR2/HMR1 ratio of approximately 70/30. (−)-MR and (+)-CLAR were prepared from HMR as described previously [2,18]. HENL (a mixture of two stereoisomers differing in stereochemistry at C-7) was prepared according to a modification of a previously described method [22]. The methods for preparation of d_6-MR and d_6-ENL, which were used as internal standards in the HPLC–MS/MS analyses, have been described previously [23,24]. END and ENL were obtained from Fluka Chemie (Buchs, Switzerland).

Ascorbic acid and *Helix pomatia*, an enzyme mixture of β-glucuronidase and sulphatase, were purchased from Biosepra (Villenouve la garenne Cedex, France). Anhydrous sodium acetate, acetic acid (HAc) (99–100%), and HPLC-grade methanol (MeoH) were obtained from J.T. Baker (Deventer, Netherlands) and sodium azide and Lichrosolv-grade methanol from Merck (Darmstadt, Germany). Polyethylene glycol (M_r 3400) was from ICN Biomedicals Inc. (Aurora, OH, USA). 4-Methylumbelliferyl-β-D-glucuronide (MUG) and 4-methylumbelliferyl sulphate (MUS) were purchased from Sigma (St. Louis, MO, USA). RP-18 material (Bondesil, 40 μm) for purification of water used in the eluent was obtained from Varian Inc. (Harbor City, CA, USA).

2.2. Animals

Forty 8-week-old male Sprague–Dawley rats were obtained from Harlan (Horst, Netherlands). The rats were housed (two animals/cage) at standardised conditions under 12 h light:12 h dark cycle at 18–21 °C and 50% relative humidity with free access to tap water and semipurified C1000 diet (Altromin Ltd, Lage, Germany).

2.3. Lignan feedings and urine collections

After an acclimatisation period of 10 days, rats were gavaged into stomach (per os) with the vehicle (50% polyethylene glycol in water containing 10% ethanol) used for the lignan administrations (200 μL per 100 g bw) and individual 24 h urine samples (“baseline” samples) were collected

in metabolic cages. The baseline group consisted of 40 animals. During the urine collection period, rats had water and C1000 diet available ad libitum. The lignan administrations were done essentially in the same way as described in our previous work [2]. The rats were randomised into four lignan administration groups: (1) HMR ($n = 11$), (2) SECO ($n = 11$), (3) MR ($n = 7$), and (4) LAR ($n = 11$). For comparison of urinary excretion of lignans, rats were gavaged per os with a vehicle containing MR, HMR, SECO or LAR in a dose of 25 mg/kg bw once per day for 10 days. The individual 24 h urine samples were collected in metabolic cages as described above after the first dose (acute exposure) and after 10 days of daily administration (sub-chronic exposure). The collection jars in the metabolic cages contained 120 μL of 0.56 M ascorbic acid and 120 μL of 0.15 M sodium azide as preservatives. The centrifuged urine volumes were measured and the samples were stored at −20 °C.

2.4. Sample preparation

The urine samples were enzymatically hydrolysed and solid-phase extracted at pH 4.0 as described previously [2] except that before extraction d_6-MR and d_6-ENL were added as internal standards in an amount of 0.97 μg and 0.54 μg, respectively. The solid-phase extracted samples were stored in MeOH at −20 °C. Just prior to HPLC–MS/MS analysis the samples were gently evaporated to dryness under a nitrogen flow at +45 °C and then redissolved in 0.5–2.0 mL of MeOH/0.1% HAc 20/80 (v/v).

2.5. Optimisation of pH during enzymatic hydrolysis

The extent of transformation of LAR to CLAR and the stability of HMR were determined at different pH values during the enzymatic hydrolysis. LAR may at least partly be converted to CLAR under acid conditions [18,25,26]. Samples of pure LAR or HMR were enzymatically hydrolysed and then solid-phase extracted as described previously [2]. The hydrolysis was done in buffer solutions at pH 3, 4, 5, and 6. LAR, CLAR, and HMR were then analysed in the samples by HPLC–MS/MS and the relative amount of LAR and HMR was determined before and after hydrolysis using d_6-MR as internal standard.

2.6. HPLC–MS/MS analyses

The HPLC system and conditions have been described previously [2]. The distilled, deionised, and filtered water was further purified using RP-18 material as described previously [2,21]. The triple-quadrupole mass spectrometer used was a Micromass Quattro Micro (Micromass, Manchester, UK) equipped with an electrospray source. Data acquisition and analyses were carried out using MassLynx version 3.5 software. Nitrogen was used as nebuliser, desolvation (flow 750 L/h), and cone gas (flow 20 L/h). Argon was used as the collision gas at a collision cell pressure of approximately 5×10^{-3} mbar. Negative ions were acquired in the multiple reaction monitoring (MRM) mode. The source block temperature was set to 150 °C, the desolvation temperature to 200 °C, and the capillary voltage at 3.40 kV. Dwell times were 0.15 s for MUS and MUG and 0.25 s for the other compounds with an interchannel delay of 0.05 s.

Table 1
Relative retention times (t_R), recorded transitions, cone voltages and collision energies used in the LC–MS/MS (MRM) analyses of the analysed compounds

Compound	t_R relative to d_6-MR	Transition (m/z)	Cone voltage (V)	Collision energy (eV)
MUG	0.65	351 → 175	22	25
CLAR	0.69	359 → 344	38	18
HMR	0.69 and 0.73	373 → 355	30	15
HENL	0.85 and 0.89	313 → 147	35	21
LAR	0.86	359 → 329	22	12
SECO	0.88	361 → 165	40	25
MR	1.00	357 → 83	38	22
d_6-MR	1.00	363 → 83	35	22
END	1.06	301 → 253	40	20
ENL	1.09	297 → 253	35	20
d_6-ENL	1.09	303 → 259	37	20
MUS	1.24	255 → 175	23	20

Individual parameters, i.e., relative retention times, recorded transitions, cone voltages, and collision cell energies used in the MRM analyses of the analysed compounds are listed in Table 1. The parameters were optimised by syringe infusion of pure compounds.

HMR and HENL were determined as a sum of the two stereoisomers.

2.7. Quantitation and quality control

Standard samples for the calibration curve were prepared by spiking pooled blank urine (collected from adult male rats fed at least 1 week with semipurified lignan-poor C1000 diet) with a stock standard solution and six dilutions of this. These samples were solid-phase extracted as the study samples, but they were not hydrolysed. The stock standard solution contained the following amounts of reference standards (μM, in MeOH): HMR 18.7, SECO 13.8, HENL 25.8, MR 14.0, LAR 20.1, END 20.5, and ENL 27.2. The following dilutions were made: 1 + 9, 1 + 19, 1 + 39, 1 + 399, 1 + 1999, and 1 + 9999 (stock solution + MeOH, v/v). The concentrations of the compounds in the most diluted standard sample ranged from 1.4 to 2.7 nM. Each standard solution contained the same amount of internal standards as the study samples. END and ENL were quantified using d_6-ENL as internal standard, the other lignans using d_6-MR as internal standard.

For each sample batch, two to nine quality control (QC) samples (i.e., the 1 + 39 dilution of the stock standard solution) were prepared in the same way as the standard samples. Furthermore, one internal control (IC) sample which consisted of pooled male rat urine collected after long-term (≥4 months) administration of HMR containing C1000 diet

(1.5%, w/w) was prepared in the same way as the individual urine samples. Deconjugation standards MUS and MUG were added to this sample to verify the successfulness of the enzymatic hydrolysis detected as the absence of MUS and MUG peaks.

The ion suppression effect on the detector response due to matrix effects was determined by analysing a standard solution (a) dissolved in pure mobile phase (MeOH/0.1% HAc 20/80, v/v) and (b) spiked into solid-phase extracted matrix (unhydrolysed pooled blank urine). Each sample was analysed three times.

2.8. Statistical analysis

All analyses were performed using Statistica version 5.1 software for Windows. The data distribution (Normality) was tested by using Shapiro–Wilk's *W*-test. Normally distributed data were analysed with one-way analysis of variance followed by Tukey's least significance test. Non-normally distributed data were analysed with Kruskal–Wallis median test followed by Mann–Whitney *U*-test. The acceptable level of significance was set at $p \leq 0.05$. Results are expressed as mean ± standard deviation.

3. Results

3.1. Analytical method

The limits of detection (LODs) and linear range of the pure lignans in MeOH/0.1% HAc (20/80, v/v) are summarised in Table 2. The LOD was defined as the concentration at which the signal-to-noise ratio was about 3.

Ion suppression due to matrix components was considerable. The recovery of the detector response was the following (%): HMR 4.41 ± 0.31, MR 4.42 ± 0.20, d_6-MR 5.25 ± 0.45, SECO 3.46 ± 0.27, LAR 3.12 ± 0.44, CLAR 2.94 ± 0.12, HENL 11.31 ± 0.76, END 6.47 ± 0.90, ENL 9.50 ± 0.31, and d_6-ENL 8.81 ± 0.16. Because of the ion suppression, the concentration of the analysed plant lignans in the most diluted standard sample was below the detection limit, whereby this point was omitted from the calibration curve.

The calibration curve linearity (*r* value) was >0.995 for all compounds in all determinations. The average accuracy of the compounds in the QC samples ranged from 85% to 93% and the average intra-assay R.S.D. in the same samples ranged from 4.3% to 10.5% (Table 2). The inter-assay variation of the lignan concentrations in the IC sample ranged from 19.6% (HENL) to 33.3% (SECO) in 11 determinations. LAR and CLAR were not present in the IC sample.

Fig. 2 shows MRM chromatograms of lignans in a spiked urine extract and Fig. 3 shows typical chromatograms of urine extracts after administration of HMR, MR, SECO, and LAR.

The measured SECO and MR content in the hydrolysed reagent blank originating from the enzyme preparation was subtracted from the SECO and MR concentrations obtained in the hydrolysed samples. The unhydrolysed pooled blank urine contained non-quantifiable amounts of ENL, MR, END, and HENL.

The solid-phase extraction recoveries were calculated by dividing the slope of the calibration curve in the presence of urine with the slope in the absence of urine and they ranged from 71% (END) to 108% (HMR).

3.2. Conversions and degradations of lignans during enzymatic hydrolysis

LAR was converted to CLAR at pH 3–5, which was observed as an increase of the relative peak area of CLAR and a decrease of that of LAR. The conversion of LAR to CLAR was 42% at pH 3, 13% at pH 4, and 1% at pH 5. HMR was stable at pH 3 and 4 (however, an isomerisation from HMR2 to HMR1 of approximately 1% could be observed) but showed a decrease at pH 5 and 6 (5% and 17%, respectively). In this work, the hydrolysis was done at pH 4.0 and the measured LAR and CLAR concentrations in the urine samples were corrected for 13% conversion of LAR to CLAR.

Table 2
Limits of detection and linear ranges in MeOH/0.1% HAc 20/80 (v/v), average accuracies and intra-assay R.S.D.s in QC samples of the analysed compounds

Compound	Detection limit (nM)[a]	Linear range (nM)[a]	Average accuracy[bd] (%)	Average intra-assay R.S.D.[cd] (%)
HMR1 or HMR2	2.02	5.07–11,210	88.4	7.0
HENL (sum of two isomers)	0.040	1.05–34,030	85.1	4.9
LAR	1.60	1.61–10,530	92.8	10.5
CLAR	0.86	0.90–19,390	92.8	4.7
SECO	0.30	0.52–27,880	90.4	6.3
MR	0.66	2.59–6700	86.5	7.4
END	0.13	0.24–13,900	92.1	4.3
ENL	0.13	0.37–23,110	88.3	5.0

[a] Injection volume 50 μL.
[b] Determined concentration/expected concentration of QC samples.
[c] Relative standard deviation of determined QC sample concentrations.
[d] Eleven determinations á $n = 2$–9 for HMR, HENL, SECO, MR, END, and ENL; two determinations á $n = 3$ for LAR and CLAR.

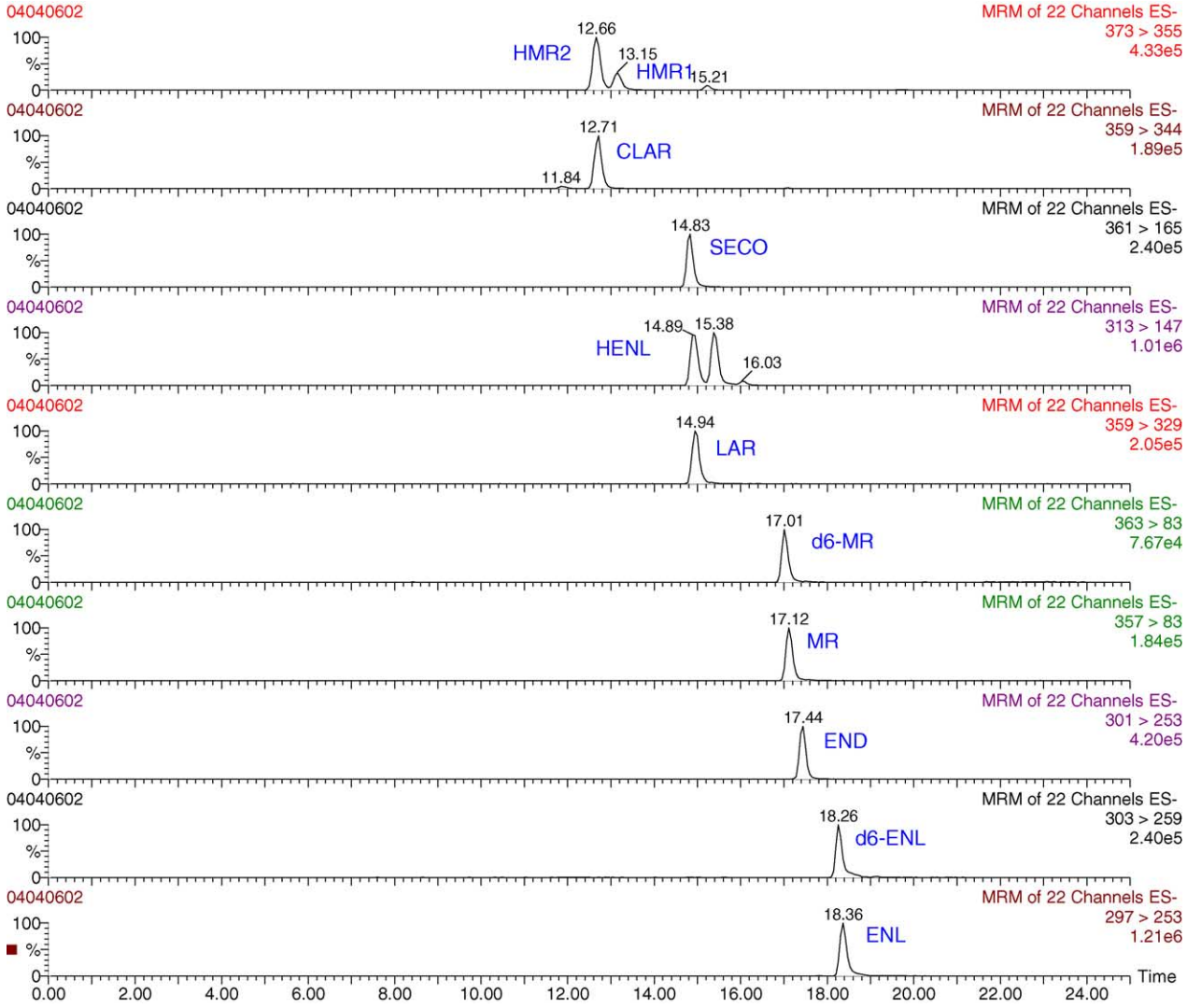

Fig. 2. MRM chromatograms of the analysed lignans in a spiked rat urine extract.

3.3. Urinary excretion of lignans

3.3.1. Mammalian lignans

The mammalian lignans END, HENL, and ENL could be detected in the urine of rats fed with the semipurified lignan-poor diet (baseline value, Table 3). They could not, however, be quantified in all the samples. HENL could be quantified in all but one sample, whereas END could be quantified in only 14 samples and ENL in 20 samples. In the other samples the concentration was below the quantitation limit (2.7 nM and 2.0 nM for ENL and END, respectively).

Administration of all the tested plant lignans (HMR, LAR, MR, and SECO) resulted in an increase of the urinary excretion of the three mammalian lignans with the exception of HENL after a single dose of SECO and LAR. The urinary quantities varied due to the time of exposure but also due to the type of the administered plant lignan. After a single dose, the total mammalian lignan excretion was similar in all groups (Table 3), whereas after 10 days the excretion was clearly higher after exposure to SECO than to the other lignans. After a single dose, HENL was the main metabolite of HMR (six rats out of 11 excreted more HENL than ENL), ENL was the main urinary lignan after administration of MR, whereas END was the main mammalian lignan metabolite of SECO and LAR (Tables 3 and 4). After 10-day exposure, ENL was the main lignan metabolite of all the tested plant lignans. The increase in ENL excretion compared to the single dose administration was 56-, 27-, 19-, and 10-fold for SECO, LAR, HMR, and MR, respectively. A significant increase in urinary END quantity could also be observed after 10 days of SECO or LAR administration, as compared to the single dose administrations (9.0- and 3.5-fold for SECO and LAR, respectively).

3.3.2. Plant lignans

Like the three mammalian lignans, also the plant lignans MR, LAR, and CLAR could be detected in the urine of rats fed with a semipurified lignan-poor diet (baseline value, Table 4).

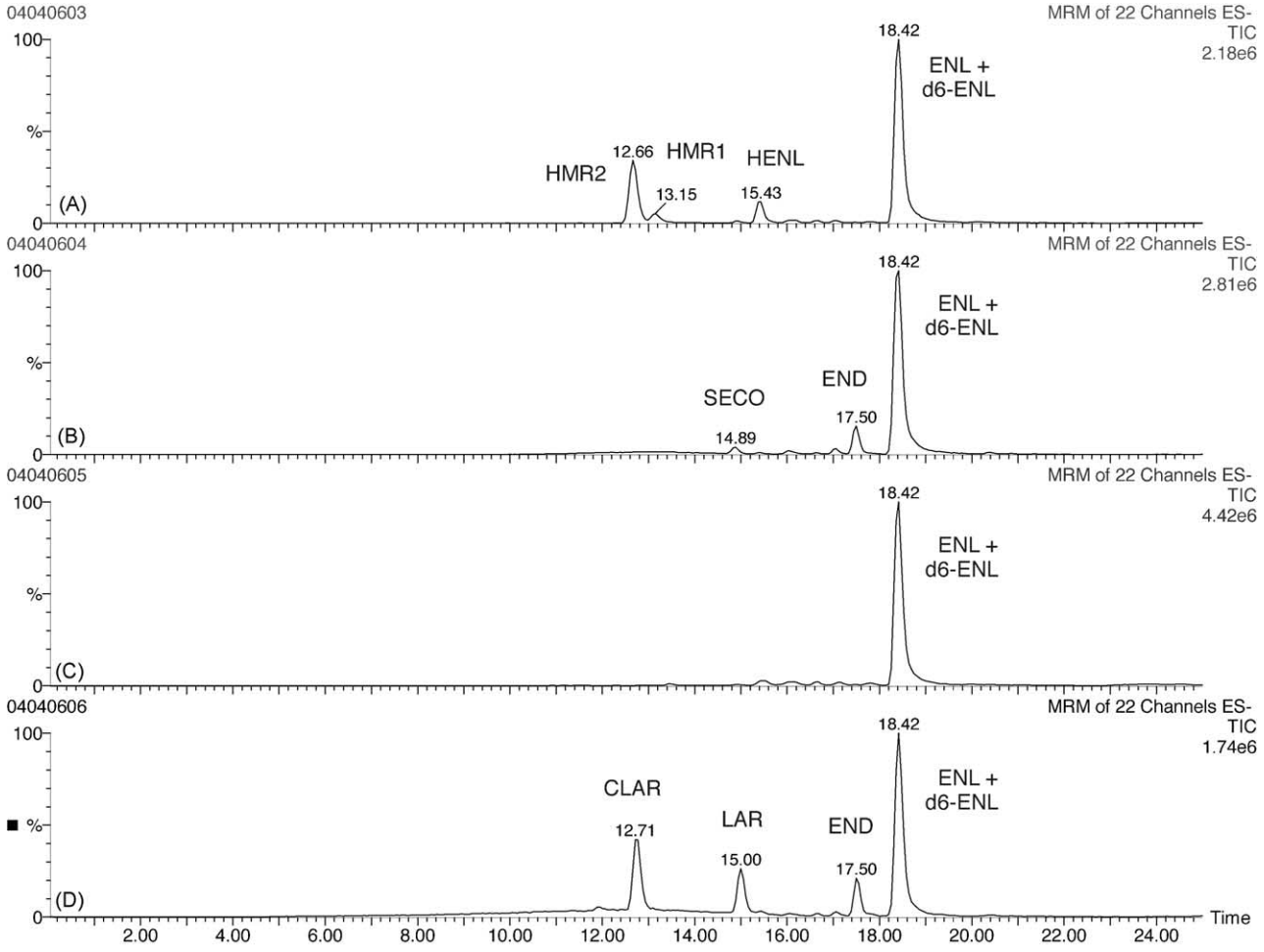

Fig. 3. Typical total ion current (TIC) chromatograms of rat urine extracts after administration of: (A) HMR; (B) SECO; (C) MR; (D) LAR.

Table 3
Baseline urinary excretion of mammalian lignans and excretion after a single oral dose (25 mg/kg bw) and after multiple doses of 25 mg/kg bw/day for 10 days of HMR, SECO, MR, and LAR

	Urinary excretion (nmol/24 h)			
	END	HENL	ENL	Total
Baseline	0.099 ± 0.26 e	0.34 ± 0.20 d	1.63 ± 2.26 e	2.06 ± 2.41 d
Administered lignan, mean dose (nmol/24 h)				
HMR				
27632 (single)	0.57 ± 0.27 d	63.4 ± 53.6 a	50.0 ± 20.5 c	114 ± 60.3 c
27738 (multiple)	13.4 ± 6.99 c,d	72.6 ± 40.8 a	950 ± 256 b	1036 ± 253 b
SECO				
27675 (single)	75.5 ± 60.2 c	0.32 ± 0.18 c,d	27.7 ± 20.6 d	104 ± 74.2 c
27696 (multiple)	675 ± 213 a	10.2 ± 5.10 b	1570 ± 601 a	2255 ± 617 a
MR				
28314 (single)	1.23 ± 1.41 d	1.34 ± 0.89 c	137 ± 62.4 c	140 ± 64.0 c
28307 (multiple)	5.85 ± 2.66 d	15.1 ± 7.51 b	1328 ± 529 a,b	1349 ± 535 b
LAR				
26768 (single)	113 ± 53.3 c	0.36 ± 0.26 c,d	33.2 ± 21.1 c,d	147 ± 69.3 c
27066 (multiple)	394 ± 205 b	11.2 ± 3.49 b	896 ± 333 b	1301 ± 442 b

Values are mean ± standard deviation. The number of individual samples available for analysis was 40 in the baseline group, 11 in the HMR and SECO administered groups, 6 in the MR administered single dose group and 7 in the multiple dose group, 10 in the LAR administered single dose group and 11 in the multiple dose group. Different letters (a–e) indicate statistically significant differences ($p < 0.05$) in concentrations of each measured compound between groups of animals administered with different lignans or with single or multiple doses of the same lignan.

Table 4
Baseline urinary excretion of plant lignans and excretion after a single oral dose (25 mg/kg bw) and after multiple doses of 25 mg/kg bw/day for 10 days of HMR, SECO, MR, and LAR

	Urinary excretion (nmol/24 h)				
	HMR	SECO	MR	LAR	CLAR
Baseline	nd	nd	0.28 ± 0.32 c	0.42 ± 0.15 e	1.58 ± 0.42 b
n_{quant}/n_{anal}	–	–	25/40	12/12	12/12
Administered lignan, mean dose (nmol/day)					
HMR					
27632 (single)	553 ± 181 b	nd	1.57 ± 1.21 b	1.08 ± 0.39 d	1.89 ± 0.72 b
n_{quant}/n_{anal}	11/11	–	11/11	11/11	11/11
27738 (multiple)	725 ± 203 a	nd	0.81 ± 0.66 b,c	2.22 ± 1.30 c	2.80 ± 1.58 b
n_{quant}/n_{anal}	11/11	–	8/11	11/11	11/11
SECO					
27675 (single)	0.072 ± 0.16 d	51.3 ± 21.3 b	0.077 ± 0.19 d	1.47 ± 0.64 c,d	2.22 ± 0.69 b
n_{quant}/n_{anal}	2/11	11/11	3/11	11/11	11/11
27696 (multiple)	nd	111 ± 42.5 a	0.20 ± 0.30 c,d	2.22 ± 1.36 c,d	2.14 ± 1.25 b
n_{quant}/n_{anal}	–	11/11	6/11	11/11	11/11
MR					
28314 (single)	1.71	nd	38.5 ± 22.4 a,b	0.55 ± 0.25 d	1.39 ± 0.47 b
n_{quant}/n_{anal}	1/6	–	6/6	6/6	6/6
28307 (multiple)	0.72 ± 1.07 d	nd	73.4 ± 87.9 a	0.50 ± 0.39 d	1.19 ± 0.75 b
n_{quant}/n_{anal}	4/7	–	7/7	5/7	6/7
LAR					
26768 (single)	2.88 ± 2.14 d	13.9 ± 8.06 c	0.26 ± 0.67 c,d	696 ± 156 b	735 ± 338 a
n_{quant}/n_{anal}	8/10	10/10	2/10	10/10	10/10
27066 (multiple)	3.71 ± 1.36 c	18.5 ± 12.9 c	0.21 ± 0.33 b,c	891 ± 222 a	863 ± 388 a
n_{quant}/n_{anal}	11/11	11/11	5/11	11/11	11/11

Values are mean ± standard deviation. nd, not detected; n_{quant}/n_{anal}, number of samples in which the lignan could be quantified/number of analysed samples. Different letters (a–e) indicate statistically significant differences ($p < 0.05$) in concentrations of each measured compound between groups of animals administered with different lignans or with single or multiple doses of the same lignan.

After administration of HMR and LAR, a considerable amount of the administered lignan was excreted into urine as such. A significant increase in HMR quantities was observed after 10 days of administration as compared to a single dose. Interestingly, the proportion of HMR2 of the total HMR amount was higher in urine both after a single dose of HMR and after administration for 10 days (78.1 ± 3.02%, $n = 22$) than that of the administered HMR preparation (70%).

LAR administration also significantly increased the excretion of CLAR (Table 4, Fig. 4). LAR and CLAR were the major urinary lignans after a single dose and still after administration for 10 days their excretion was almost as high as that of ENL. Furthermore, after a single dose the quantity of CLAR was significantly higher than that of END or ENL. However, only slightly (but significantly) increased quantities of SECO or MR were observed after the administration of the parent compounds.

Administration of HMR, LAR, MR or SECO also changed the urinary excretion of plant lignans other than gavaged. In addition to the high CLAR excretion, LAR administration also increased the SECO and HMR excretion as compared to the baseline urine samples ($p < 0.05$) (Table 4, Fig. 4). Further, a slightly increased urinary concentration of LAR was detected after HMR and SECO administration ($p < 0.05$). Administration of HMR increased significantly the excretion of MR after a single dose, and 10 days of MR administration increased the excretion of HMR as compared to the baseline urine quantity ($p < 0.05$), suggesting possible transformation of plant lignans into each other.

4. Discussion

4.1. Analytical method

The linear range of the developed method was wider for mammalian lignan metabolites HENL and END than in our previous method [21]. The linear range extended over four orders of magnitude for HENL, END, and ENL and also for the plant lignan SECO. The linear range for the other lignans exceeded three orders of magnitude. Due to large individual differences in plant and mammalian lignan excretion, a wide analysis range is an important advantage of this developed analysis method.

The LODs were higher than in the previous method [21], which was mainly due to the more sensitive instrument used in that study. Furthermore, the LOD of ENL was approximately the same as what has been achieved using the time-resolved fluoroimmunoassay method [27] and lower for END and ENL than those achieved using HPLC with coulometric array detection [28].

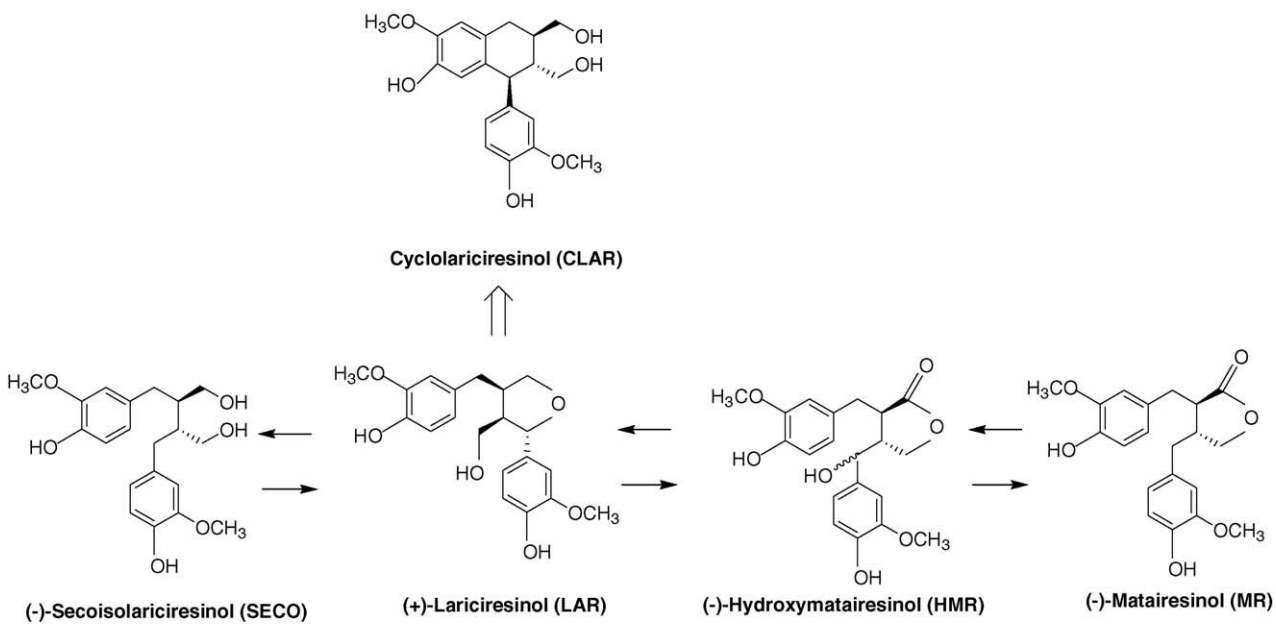

Fig. 4. Metabolic transformations of plant lignans. The arrows indicate a measured significant increase ($p < 0.05$) in urinary excretion after administration of the lignan in question.

Despite the extensive ion suppression effect due to matrix components the method is usable, as the variability in the composition of the rat urine is expected to be small. The urine is collected from a relatively large group of animals of the same strain, age, and sex that have been housed at standardised conditions and fed the same diet. The usability of the method is further supported by low intra-assay R.S.D.s and good accuracy of the QC samples.

4.2. Urinary excretion of lignans and metabolic conversions

4.2.1. Types and quantities of excreted mammalian lignans

The conversion of some pure plant lignans to mammalian lignans in rats has been shown in previous studies. SECO [1,2,6] and SDG [4,5] have been demonstrated to produce both END and ENL, whereas MR produces mainly ENL [1,2]. In an in vitro fermentation study with human fecal microbiota, SECO and LAR were demonstrated to produce END and ENL [7]. The present study shows that LAR is converted to END and ENL also in vivo, however, a large portion of LAR is converted to CLAR probably because of the acid conditions in the stomach. In rats, HMR has been shown to be metabolised to ENL [2,3] and HENL [3] and to a small degree also to END [2,3,15], which is supported by the results of the present study. This study also shows that administration of MR, SECO or LAR leads to an increased excretion of HENL, however, in the case of SECO or LAR only after prolonged exposure. In one study, Niemeyer et al. [6] could not detect any aliphatic hydroxyenterolactones at all in rat urine after a single dose administration of SECO or MR, but in another study [29], they detected traces of such compounds in the urine of untreated rats and elevated levels in the urine of rats fed with flaxseed or dosed with ENL.

Interestingly, in our present and in previous studies, a slightly, but significantly, elevated urinary excretion of END was observed after HMR administration [2,15] and MR administration as well as after ENL administration [2]. In in vitro fermentations with human fecal microbiota, HMR has been shown to produce a trace amount of END [7], but no END formation from MR has been reported [7,8] and in studies with liver microsomes no END formation from ENL has been reported [30]. In our studies the excretion of END after HMR, MR, and ENL administration has been very small and it may be below the detection limit using more insensitive analytical methods. The formation of END from MR and HMR may go via ENL. Small amounts of END may be formed via a reduction of the lactone ring carbonyl group of ENL.

In our previous study, the quantities of ENL and END excreted in 24-h rat urine samples were compared after administration of a single per os dose of equivalent amounts of the plant lignans SDG, SECO, MR or HMR [2]. The results showed that the quantities varied after exposure to the different lignans. Administration of MR resulted in the highest ENL excretion, while the total quantity of mammalian lignans was highest after administration of SECO. In the present study after a single dose the results were similar. In a previous study in which HMR was administered to rats for 2 days in different doses (3–50 mg/kg bw), hydroxyenterolactone was found in urine in much smaller amounts than ENL [3], whereas in the present study the mean HENL excretion was higher than the ENL excretion after a single dose. However, due to lack of authentic reference compounds, the analysis was only semi-quantitative in the previous study.

The present study shows that a longer period of lignan exposure affects the quantity of excreted mammalian lignans. The excretion of all three mammalian lignans increased significantly after prolonged exposure to all the tested lignans with the exception of HENL after HMR dosage. ENL showed the highest increase, i.e., 10–56-fold compared to a single-dose administration. Of the tested plant lignans, SECO showed the highest increase in total mammalian lignan excretion after prolonged exposure compared to the single dose administration (22-fold compared to approximately 9-fold for the other three lignans).

Only a minor portion of the administered lignan was excreted into 24 h urine as mammalian lignan metabolites. After a single dose of the plant lignan, the proportion of excreted amount of enterolignans (sum of END, HENL, and ENL) of the administered amount ranged from 0.38% (SECO administration) to 0.54% (LAR administration). After 10 days, the excreted portion of enterolignans ranged from 3.8% (HMR administration) to 8.2% (SECO administration). These figures can be calculated by dividing the total mammalian lignan excretion with the mean lignan dose in Table 3.

Differences in the basal diet may also have an impact on the lignan metabolism of the rats. In our previous study [2] open formula RM1 chow diet which is rich in plant fiber materials was used as the basal diet, whereas in this study semipurified lignan poor C1000 diet was used. As expected, the RM1 diet caused a higher baseline excretion of END and ENL (0.29 and 37 nmol/24 h, respectively) than in this study (0.09 and 1.6 nmol/24 h, respectively, Table 3). Differences in mammalian lignan excretion after lignan administration in the two studies can also be observed, especially with SECO. In the previous study, SECO produced much larger amounts of END and ENL than in this study.

4.2.2. *Metabolism of plant lignans*

Some of the plant lignans are also excreted into urine as such which raises the question about the putative health effects of plant lignans. In the present and in a previous study [2], significant differences in excretion of the plant lignans as such were observed. Administration of HMR increased the urinary quantity of HMR considerably more than what was seen in SECO or MR quantities after administration of an equivalent dose of SECO or MR. The higher HMR2/HMR1 ratio observed in urine as compared to the administered HMR preparation indicates either isomerisation of HMR1 to HMR2 in the body or that HMR1 is more easily metabolised than HMR2. Administration of LAR resulted in a high excretion of both LAR and CLAR, which were the major urinary lignans after a single dose administration. The conversion of LAR to CLAR in vivo may at least partly take place under the acid conditions prevailing in the stomach and/or in the upper parts of the gastrointestinal tract, as it is known that LAR is converted to CLAR under acid conditions in vitro [18,26,27]. It is also possible that LAR is partly converted to CLAR in the bladder or during urine collection, but very recent experiments in our laboratory show that large amounts of CLAR can also be found in the serum of rats orally administered with LAR, which confirms that CLAR is a real major metabolite of LAR.

Examples of transformations of the ingested plant lignan to other plant lignans could be observed as shown in Fig. 4. HMR dosage leads to a significant increase in the excretion of LAR, which also involves a reduction of the lactone ring carbonyl group like in the formation of END from ENL (discussed in Section 4.2.1). In this study, administration of SECO increased the excretion of LAR and MR administration increased the excretion of HMR, although the quantities compared to the major metabolites were small. In vitro in rat liver microsomes SECO was also transformed to LAR and MR to HMR [6], suggesting the possibility for P450 mediated reactions. However, in rats these metabolites were not detected in urine after administration of SECO or MR [6]. The amounts of identified oxidative metabolites in rat urine were very small compared to other metabolites [6]. It is thus possible that the amounts of some of these oxidative metabolites were below the detection limits. Therefore, we cannot exclude the possibility that part of the observed plant lignan transformations in this study are caused by P450 mediated reactions.

5. Conclusions

This study shows that a longer time period of plant lignan exposure affects both the type and the quantity of both plant and mammalian lignans excreted in urine. Prolonged exposure significantly increases the urinary excretion of the mammalian lignan ENL compared to a single dose administration. Of the studied lignans, SECO produced the highest amounts of mammalian lignans in total. Furthermore, this study indicates that plant lignans may be transformed to each other in vivo.

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