THE EXCRETION OF LIGNANS IN RATS – EVIDENCE FOR AN INTESTINAL BACTERIAL SOURCE FOR THIS NEW GROUP OF COMPOUNDS

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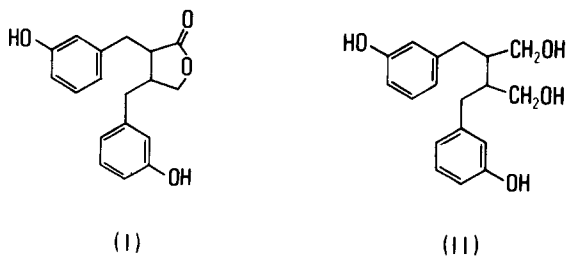
1. Introduction

The lignans are a class of compound with a dibenzylbutane skeleton [1] and which until recently had only been found in higher plants [2]. The first lignans to be identified in humans and monkeys were described in [3,4], their structures differing from all known plant lignans in having *meta*-substitution in the benzyl groups. The two principal lignans identified were 2,3-*bis*-(3′-hydroxybenzyl)-butyrolactone (HBBL) and 2,3-*bis*-(3′-hydroxybenzyl)-butane-1,4-diol (HBBD) and their structures are shown in fig.1. The quantitative excretion of HBBL and HBBD was found to exhibit a cyclic pattern during the menstrual cycle of humans [3–6] and monkeys [7], maximum excretion occurring in the luteal phase and elevated levels were found in urine collected during early pregnancy [6]. Circumstantial data suggested lignans were possibly of ovarian origin or their production was modulated by ovarian function [3]. HBBL was also identified in human pregnancy and suggested to be of ovarian source or produced by ovarian stimulus [8].



(I) (II)

Fig.1. Chemical formulae of the principal mammalian lignans: (I) 2,3-*bis*-(3′-hydroxybenzyl)-butyrolactone; (II) 2,3-*bis*-(3′-hydroxybenzyl)-butane-1,4-diol.

To facilitate studies on the biosynthesis and metabolism of lignans, a search was made for an inexpensive and convenient laboratory animal. Here we report on the excretion of lignans in the urine and bile of rats, demonstrate that they are formed by intestinal bacteria and confirm an enterohepatic circulation.

2. Experimental

2.1. *Animal experiments*

Rats were placed in restraining cages with free access to water or saline and food pellets. Bile and urine were collected for 1 week at 12–24 h intervals from conventional adult male and female rats (~200 g) of the Sprague-Dawley strain. Bile fistula were inserted into rats by conventional procedures and collections of bile commenced from the day of operation. A number of rats, with and without bile fistula, were ovariectomised and collections of bile and/or urine started 1 week after the operation. Urine was collected over a 1 week period from 3 adult male rats of the Long-Evans strain, reared germ-free as in [9] and placed in metabolism cages with free access to water and a sterilized diet. Urine collected from 3 adult male rats of the same stock, reared outside the germ-free isolators, on the same diet, were used as controls. Collections (12 h) were made from 3 adult female germ-free rats of the Sprague-Dawley strain.

Blood samples were obtained from the inferior vena cava and the portal vein of female Sprague-Dawley rats, transferred to heparinised tubes and centrifuged immediately. The plasma was separated and stored at –20°C until analysed.

2.2. *Analytical procedure*

2.2.1. Total lignans

Lignans and their conjugates were extracted from urine using Sep-Pak-(C_{18}) cartridges essentially as described for steroids [10]. Lignan conjugates were hydrolysed by *Helix pomatia* digestive juice (a mixed β-glucuronidase and sulphatase preparation) and the deconjugated lignans were extracted from the hydrolysate using Sep-Pak (C_{18}) cartridges. The extracts were then filtered through small columns of a cation exchange gel, SP-Sephadex-LH-20 [H^+] packed in methanol and then purified on small columns of a strong anion exchange gel, triethylaminohydroxypropyl Sephadex LH-20 (TEAP-LH-20) in the OH^- form. Neutral compounds were eluted with methanol and methanol/chloroform (1:1, v/v) prior to the selective elution of phenolic compounds with methanol saturated with CO_2 [11]. Lignans were analysed by gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS) as their trimethylsilyl (TMS) ether derivatives [6].

2.2.2. Analysis of individual lignan conjugates in urine, bile and plasma

This was done by the procedures in [11,12]. After extraction, lignans were separated according to their mode of conjugation, into unconjugated phenolic compounds, glucuronides, monosulphate and disulphate conjugates on small columns of the anion exchange gel TEAP-LH-20. Lignan glucuronides were hydrolysed enzymatically and the sulphates solvolysed, before purification on small columns of TEAP-LH-20 [12].

2.3. *GC and GC–MS analysis*

Following addition of an internal standard 3α-hydroxy-5β-cholestane, the lignans were converted to TMS ether derivatives and analysed by capillary column GC–MS [11,12]. Quantification was performed by GC on open-tubular glass capillary columns coated with SE-30 and using flame ionisation detection [12]. Due to the low concentration of lignans in the sulphate fractions and in plasma, quantification was carried out using a selective ion monitoring GC–MS procedure as in [12].

3. Results and discussion

3.1. *Identification of lignans in urine and bile from conventional rats*

Two lignans, isolated from the urine and bile of conventional rats were identified definitively as 2,3-*bis*-(3′-hydroxybenzyl)-butyrolactone (HBBL) and 2,3-*bis*-(3′-hydroxybenzyl)-butane-1,4-diol (HBBD) on the basis of GC retention times (t_R = 27.50 MU and t_R = 27.70 MU, respectively for the TMS ethers fig.2), complete mass spectra and partial mass spectra obtained from fragment ion current (FIC) chromatograms constructed of characteristic ions given by lignan derivatives (fig.3). These two lignans are identical to the lignans found in the urine of humans [3,4,8] and vervet monkeys [7]. A phenolic compound (referred here as 386/192 t_R = 27.3 MU) of apparently related structure was found in all samples in greater amounts than both of the identified lignans (fig.2,3). The mass spectrum of the TMS ether of this compound is shown in fig.4 and indicates a molecular ion at *m/z* 386. The fragment *m/z* 207 is formed by the loss of 179 mass units from the molecular ion, due to cleavage of a benzylic group containing a trimethylsiloxy function, a characteristic fragmentation seen in the mass spectra of HBBL and HBBD [3,4]. The most prominent ion

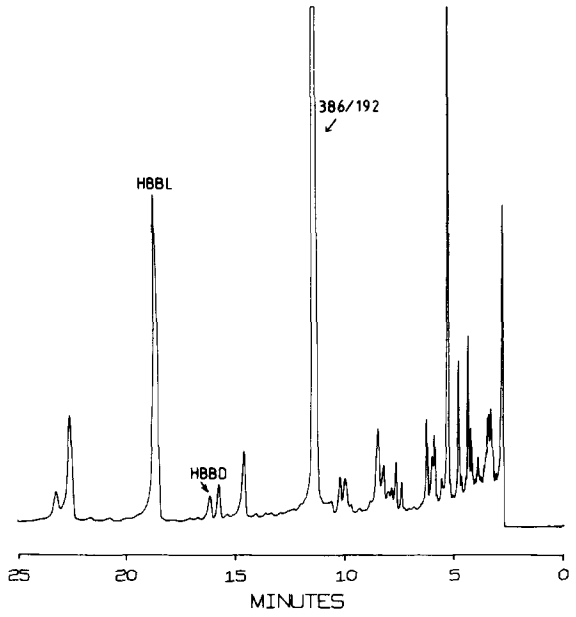


Fig.2. A typical isothermal gas chromatographic recording of the TMS ether derivatives of the compounds present in a phenolic glucuronide fraction from the urine of a female rat.

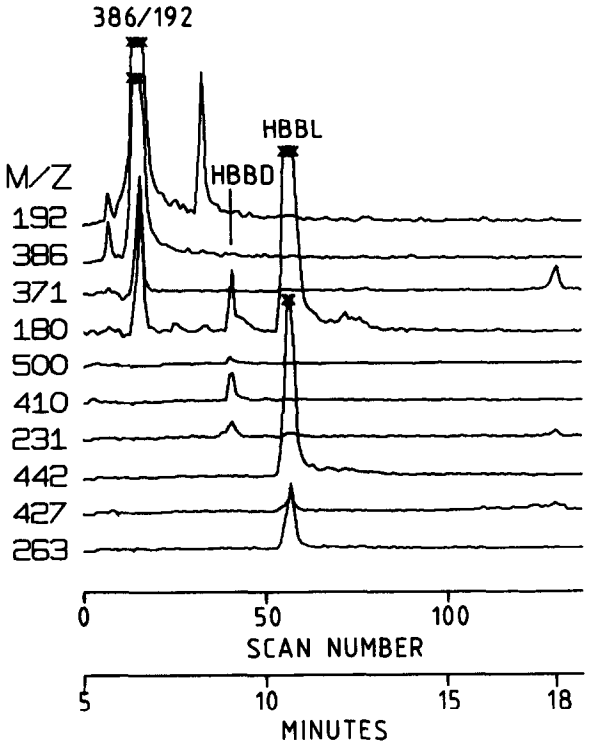


Fig.3. GC–MS analysis of lignans in the phenolic glucuronide fraction from the urine of a normal female rat. FIC chromatograms constructed by the computer are shown for the ions characteristic of TMS ethers of the unidentified compound 386/192 (*m/z* 192, 386 and 371), HBBD (*m/z* 180, 500, 410 and 231) and HBBL (*m/z* 180, 442, 427 and 263).

and base peak in the spectrum, *m/z* 192, differs from the base peak in the TMS ethers of the lignans, (*m/z* 180). In view of the phenolic nature of this compound, this ion may arise from a fragment consisting of $(CH_3)_3Si–O–C_6H_4–CHCH_2$, but this remains to be established. This ion is however known to be produced by certain phenolic compounds [13]. The presence of two reactive hydroxyl groups was established from analysis of the mass spectrum of the deuterium labelled TMS ether derivative. When chromatographed on the weak anion exchanger DEAE–Sephadex, this compound was eluted in the fraction corresponding to diphenolic compounds, confirming the two derivatisable hydroxyl functions to be aromatic. As the mol. wt of this compound is 386 (as the *bis*-TMS ether derivative) its structure must differ from the lignans HBBL and HBBD in not having the basic dibenzylbutane skeleton. Compound 386/192 has also been found in small quantities in the urine of humans, and further work is ongoing to establish the structure of this compound.

3.2. *Quantitative excretion of lignans in urine and bile*

3.2.1. Conjugation

The diphenolic compounds described above were excreted in urine and bile almost exclusively as acidic conjugates. These conjugates were identified as glucuronides and sulphates from their mobilities on the ion exchange gel TEAP-LH-20 and because they could be hydrolysed by β-glucuronidase enzymes and by a mild solvolytical procedure, respectively [12]. HBBL and HBBD have previously been definitively identified in human urine as intact glucuronides using GC–MS and quantitatively are excreted in urine almost exclusively in this form [12].

In rat urine the distribution of the lignans HBBL and HBBD within the conjugate classes (table 1) was similar to the human. One difference from the human and monkey, is the higher proportion of sulphate conjugates present in rat urine, however rats have a high level of sulphokinase activity in the liver [14]. Although not yet proven, it was suggested that this

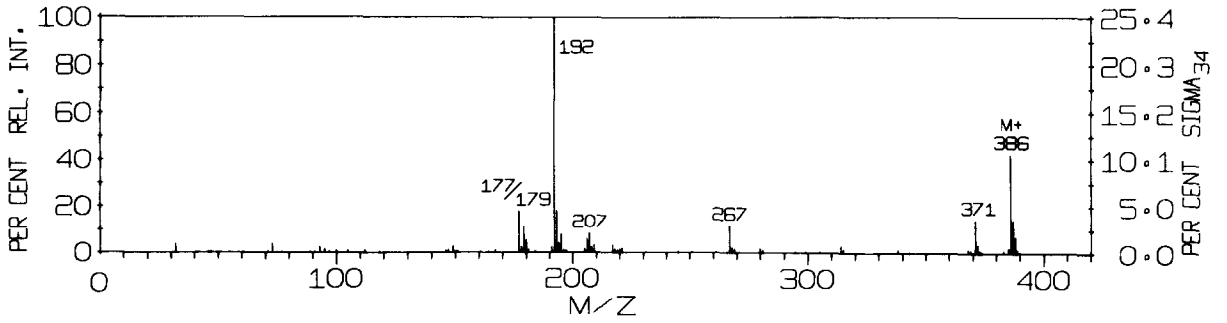


Fig.4. Mass spectrum of the TMS ether derivative of the unknown diphenolic compound 386/192, isolated from rat urine.

Table 1
Distribution (%) of conjugates of the lignans 2,3-*bis*-(3'-hydroxybenzyl)-butyrolactone (HBBL), 2,3-*bis*-(3'-hydroxybenzyl)-butane-1,4-diol (HBBD) and an unknown diphenolic compound (386/192) in urine and bile from conventional rats

Fluid	Sex	Glucuronides (%)			Monosulphates (%)			Disulphates (%)		
		HBBL	HBBD	386/192	HBBL	HBBD	386/192	HBBL	HBBD	386/192
Urine	Male	74.9	91.7	99.7	3.9	2.5	0.1	21.2	5.8	0.2
Urine	Female	90.7	95.2	99.9	3.0	1.9	0.1	6.3	2.9	0.1
Bile	Female	99.0	97.5	99.5	0.8	2.4	0.2	0.1	0.1	0.2

conjugation most probably occurs in the liver [12]. In rat bile all 3 compounds were identified as glucuronide conjugates (table 1) and only traces of sulphates (<1%) were found. The unknown diphenolic compound differed from the 2 lignans in being present exclusively as a glucuronide conjugate in both urine and bile (>99.5%).

3.2.2. Conventional rats

All 3 diphenolic compounds were excreted in urine and bile in relatively large amounts by normal rats. On a body weight basis the daily urinary excretion of the lignans HBBL and HBBD considerably exceeded (5–20-fold) the amounts found in humans [3,5,6] and there was some evidence to indicate a sex difference in the excretion of these compounds; female rats excreting larger amounts of HBBL and smaller amounts of the unknown diphenolic compound compared with male animals. Such differences are not uncommon in rats, steroid metabolism is characterised by qualitative differences between the male and female [15], however, in view of the limited number of measurements, additional data are required to confirm this observation. In all cases, the quantitative urinary excretion of the unknown diphenolic compound was always greater than the lignans HBBL and HBBD and its behaviour was found to parallel that of the 2 lignans.

Earlier studies in the human suggested the ovaries as a possible source of lignans [3,6,8]. This hypothesis was tested by measuring the excretion of lignans before and after the removal of the ovaries from normal rats. No significant difference between the urinary excretion of these compounds before and 7 days after ovariectomy was found (table 2).

Table 2
Total excretion (μg/24 h) of the lignans 2,3-*bis*-(3'-hydroxybenzyl)-butyrolactone (HBBL) and 2,3-*bis*-(3'-hydroxybenzyl)-butane-1,4-diol (HBBD) and the unknown diphenolic compound 386/192 in the urine and bile of rats under different conditions

Body fluid	Sex	Conditions	Total excretion (μg/24 h)		
			HBBL	HBBD	386/192
Urine	Male	Normal	4.0	1.0	128.6
Urine	Female	Normal	21.2	1.5	50.0
Urine	Female	Bile drainage	6.0	0.4	12.0
Bile	Female	Bile drainage	14.0	0.4	60.4
Urine	Female	Ovariectomized	20.2	0.8	46.8
Urine	Female	Ovariectomized + bile drainage	10.3	0.4	18.2
Bile	Female	Ovariectomized + bile drainage	11.0	0.3	57.2
Urine	Male	Germ-free	<0.1	<0.1	<0.1
Urine	Female	Germ-free	<0.1	<0.1	<0.1

Table 3
Concentrations (ng/ml plasma) of 2,3-*bis*-(3'-hydroxybenzyl)-butyrolactone (HBBL) and compound 386/192 in venous blood from a female rat

Blood source	Conditions	HBBL		386/192	
		Free	Glucuronide	Free	Glucuronide
v. cava inf.	Normal	1.1	3.9	3.9	63.0
v. porta	Normal	2.8	56.0	23.6	351.0
v. porta	Bile drainage[a]	1.3	1.6	7.4	5.4

[a] Blood collected 2 days after insertion of a bile fistula

HBBL, HBBD and compound 386/192 were identified in high concentrations in rat bile. When bile fistula were inserted in female rats and the bile drained, the daily urinary excretion of all three compounds decreased significantly (table 2). The quantitative excretion of HBBL and the unknown compound in bile during this period was 2–5-fold greater than in the corresponding urine samples, while the combined excretion of these compounds in urine and bile was similar to the urinary excretion by intact conventional rats. All of this evidence indicates that an enterohepatic circulation exists which is further substantiated by measurements of the concentrations of HBBL and compound 386/192 in samples of plasma collected from the portal vein (table 3). Concentrations of conjugated HBBL (56 ng/ml) and compound 386/192 (351 ng/ml) in plasma were very much higher than in the inferior vena cava (3.9 ng/ml and 63 ng/ml, respectively, for both compounds).

In the portal vein, these compounds were present principally as glucuronide conjugates which indicates that they are efficiently re-absorbed from the gut as conjugates. As would be expected, in response to interruption of the enterohepatic circulation, the concentrations of conjugated HBBL and compound 386/192 in the portal vein were reduced dramatically on biliary drainage.

3.2.3. Germ-free rats

The highly aromatic nature and unusual structure of the lignans together with optical rotation measurements showing their occurrence as racemic compounds [4,8] was an early indication that these compounds could be formed by intestinal microflora. To confirm this hypothesis urine was collected from male and female rats, reared germ-free [9]. Lignan excretion under these conditions was undetectable (<0.1 μg/day) as was the excretion of the unknown diphenolic compound, thereby confirming a bacterial origin for the production of this new class of compounds.

The formation of lignans in the intestine was supported by the observation that biliary drainage resulted in little change in the blood levels of unconjugated lignans whereas levels of lignan glucuronides decreased dramatically in the portal vein (table 3). The unconjugated compounds therefore must represent newly formed phenolic compounds in the intestine which are returned to the liver for conjugation to glucuronic acid before being excreted in the bile. These results also indicate that there occurs an efficient reabsorption of the glucuronide conjugates from the intestine.

4. Conclusions

The data presented indicate the rat to be a convenient animal model for the study of lignans, a new group of compounds recently identified in humans. In addition to the previously identified lignans, 2,3-*bis*-(3'-hydroxybenzyl)-butyrolactone (HBBL) and 2,3-*bis*-(3'-hydroxybenzyl)-butane-1,4-diol (HBBD) a compound of an as yet unidentified structure is quantitatively the major diphenolic compound excreted by rats and its physiological behaviour was found to parallel that of the lignans.

The observations of a cyclic pattern of excretion of HBBL and HBBD during the menstrual cycle of humans [3,5,6,8] and monkeys [7] suggested initially that they were produced by the ovaries or influenced by ovarian function. Using the rat however, we have unequivocally demonstrated that lignans are not formed in the ovaries but are biosynthesised by intestinal bacteria, resembling therefore vitamin K synthesis. Studies using selective antibiotic therapy in

humans have also confirmed a bacterial source for the production of lignans [16]. An enterohepatic circulation of lignans occurs; they are absorbed from the intestine, transferred to the liver and conjugated, excreted in bile and reabsorbed mainly as conjugates. Their physiological behaviour resembles cholesterol [17,20], bile acids [21] bile pigments [22], oestrogens [23,24], vitamin B_{12} [25] and folic acid [26,27], compounds which also have an enterohepatic circulation.

Acknowledgements

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Note added in proof

Using GC–MS and NMR spectrometry the unknown compound 386/192 described has been identified as equol (Axelson, M., Kirk, D. N., Lawson, A. M. and Setchell, K. D. R., in preparation), a compound first isolated from pregnant mare's urine by G. F. Marrian and G. A. D. Haselwood (Biochem. J. (1932) 26, 1227) and later shown to possess weak oestrogenic activity (Schutt, D. A. and Brayden, A. W. (1968) Aust. J. Agric. Res. 19, 545).

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