CONJUGATION OF LIGNANS IN HUMAN URINE

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1. Introduction

The definitive identification of two new lignans, with the structures 2,3-bis-(3′-hydroxybenzyl)-butyrolactone and 2,3-bis-(3′-hydroxybenzyl)-butane-1,4-diol isolated from the urine of humans and several animal species, has been reported [1,2]. The quantitative urinary excretion of the dibenzybutyrolactone has been shown to peak during the luteal phase of the menstrual cycle of women [1,3] and the vervet monkey [4] and in pregnancy maximum excretion of this compound occurred between weeks 14–22 of gestation [3]. The urinary excretion of these compounds by men is quantitatively similar to that in the follicular phase of normal ovulating women [3].

These compounds are almost exclusively excreted in urine as acidic conjugates [1,4] and we show using a combined liquid chromatography and gas chromatography–mass spectrometric (GC–MS) method, developed for the analysis of steroid conjugates [5], that these conjugates are principally glucuronides.

2. Experimental

2.1. *Urine samples*

Daily urine collections were obtained from healthy male and female human subjects (aged 6–62 years) and stored at −20°C immediately after collection to minimise possible bacterial degradation and artefact formation.

2.2. *Extraction and conjugate separation*

Complete details and the evaluation of the techniques appear in [5]. Lignans were extracted from urine samples on a column of Amberlite XAD-2 (Rohm and Haas, Philadelphia, PA). The extract was then passed through a column of cation exchange gel, SP-Sephadex C-25 (Pharmacia Fine Chemicals, Uppsala) prior to separating the lignans according to their mode of conjugation on a small column of the lipophilic strong anion exchange gel triethylamino-hydroxypropyl Sephadex LH-20, TEAP-LH-20 [5]. Neutral compounds were eluted from the column with aqueous methanol. The stepwise elution of unconjugated phenolic compounds, glucuronide conjugates, monosulphate and disulphate conjugates, was achieved with the solvents 72% aqueous methanol saturated with carbon dioxide, 0.4 M formic acid, 0.3 M acetic acid–potassium acetate (pH 6.5) and 0.5 M potassium acetate (pH 10), respectively [5].

2.3. *Unconjugated lignans*

Those isolated in the free phenolic fraction were converted to trimethylsilyl (TMS) ether derivatives and analysed by GC.

2.4. *Lignan glucuronides*

These were analysed as intact derivatives and after removal of the glucuronic acid moiety. The former analysis was performed after methylating the carboxyl group of the glucuronide with diazomethane (under these conditions the free phenolic hydroxyl group of the lignan glucuronide does not react [2]) and purifying the methyl ester derivatives, dissolved in methanol, on a column of TEAP-LH-20 gel. After washing the column with methanol the conjugated lignan derivatives (which possess a phenolic group) were eluted by methanol saturated with carbon dioxide. The hydroxyl groups of the lignan glucuronide methyl esters were then convered to TMS ether derivatives and analysed by GC–MS.

2.5. *Deconjugation of the glucuronide conjugates*

Enzymatic hydrolysis was achieved using two different β-glucuronidase preparations. The dried sample was dissolved in 5 ml 0.2 M sodium acetate buffer (pH 4.5). Enzyme was then added, 1 mg β-glucuronidase purified from bovine liver equivalent to 10 000 Fishman Units (Sigma St Louis, MO) or 0.3 ml *Helix pomatia* digestive juice equivalent to 30 000 Fishman Units (L'Industrie Biologique Francaise, Gennevilliers) and the sample incubated at either 37°C for 24 h or at 62°C for 1 h, respectively. The liberated lignans were then extracted on Amberlite XAD-2 and purified on TEAP-LH-20 as in [4,5].

Removal of the glucuronic acid moiety was also performed by oxidative cleavage with periodate [6] which produces formate esters of the glucuronides. After reaction the mixture was diluted with water and the products extracted by ethyl acetate. The solvent was evaporated, the products redissolved in methanol and purified on a column of TEAP-LH-20 gel. During this purification procedure, hydrolysis of the formate esters occurs on this basic ion exchanger [7]. The free lignans obtained were converted to TMS ether derivatives and analysed by GC–MS.

2.6. *Lignan sulphates*

The mono- and disulphate fractions were solvolysed in acidified tetrahydrofuran (4 mM sulphuric acid) [5,8]. The liberated lignans were then purified on small columns of TEAP-LH-20 [5] and TMS ether derivatives prepared.

2.7. *Preparation of TMS ether and deuterium labelled TMS ether derivatives*

TMS ethers were prepared by addition of 100 μl pyridine:hexamethyldisilazane:trimethylchlorosilane (3:2:1, by vol.) and heating at 60°C for 1 h. The silylation reagents were removed under a stream of nitrogen and the derivatives redissolved in hexane.

Deuterium (d_9)-labelled TMS ethers were prepared by addition of 100 μl trimethyl-$^2H_9$-chlorosilane: pyridine (20:1, v/v) using the above conditions.

2.8. *Gas chromatography*

GC was done on a Pye 104 gas chromatograph equipped with a flame ionisation detector and housing a 20 m × 0.3 mm open-tubular glass capillary column coated with SE-30 [9]. Samples were introduced via an all-glass, solid injection system [10]. Nitrogen was the carrier gas with an inlet pressure of 50 kPa, giving a flow rate through the column of ~1 ml/min. The oven temperature was 255°C.

2.9. *Gas chromatography–mass spectrometry*

GC–MS analysis was done on a modified LKB 9000 instrument [11] housing an open-tubular glass capillary column (25 m × 0.3 mm) coated with SE-30, heated at 250°C and connected to the ion source via a single stage adjustable jet separator [12].

The derivatised lignan glucuronides were chromatographed on a conventional 1.5% SE-30 packed column (1 m × 3.4 mm) at 255°C. Temperatures of the molecular separator and the ion source were 275°C and 290°C, respectively; energy of bombarding electrons, 22.5 eV; ionising current, 60 μA and accelerating voltage, 3.5 kV. Repetitive magnetic scanning (usually 8–10 scans/min) over the mass/charge (m/z) range 0–800 amu, was initiated after a suitable delay from the time of sample injection. Methods for the computerised evaluation of the mass spectral data have been described [13]. Quantification with higher sensitivity was obtained by operating the instrument in a single ion monitoring mode, recording the ion of m/z 180.

2.10. *Identification and quantification of lignans*

Identification was based upon the GC retention time, the complete mass spectrum and partial mass spectra obtained from fragment ion current (FIC) chromatograms constructed of characteristic ions given by the lignan derivatives. An estimation of the amount of lignans present in the hydrolysed glucuronide fractions was obtained by comparison of the peak area response with the peak area obtained from a known amount of internal standard, 5β-cholestan-3α-ol added prior to derivatisation.

3. Results and discussion

3.1. *Identification of lignan conjugates*

Circumstantial evidence for the existence of glucuronide conjugates of the lignans, 2,3-bis-(3′-hydroxybenzyl)-butyrolactone and 2,3-bis-(3′-hydroxybenzyl)-butane-1,4-diol, was obtained from their chromatographic mobilities on the lipophilic strong anion exchanger, TEAP-LH-20, and from the finding that these lignans were hydrolysed by β-glucuronidase preparations [4]. Oxidation with periodate and mild

alkali treatment also resulted in formation of the free lignans, reactions that would be expected for glucuronide conjugates [7]. Furthermore, when the Me ester–TMS ether derivatives of the intact lignan glucuronides were analysed by GC–MS, their retention times on a SE-30 column were 13–14-times longer than the corresponding TMS ether derivatives of the free compounds. Similar differences in retention times have been reported for Me ester–TMS ether derivatives of bile acid glucuronides [14,15].

Definitive evidence for the occurrence of these lignans in urine as glucuronide conjugates was obtained by GC–MS. The mass spectra of the Me ester–TMS ether derivative (upper panel) and the Me ester–deuterated TMS ether (lower panel) derivative of the glucuronide conjugates of 2,3-bis-(3′-hydroxybenzyl)-butyrolactone are compared in fig.1. In common with the mass spectra of derivatives of glucuronide conjugates of oestrogens [16] the intensity of the molecular ion (M^+) *m/z* 776 in the Me ester–TMS ether of this lignan glucuronide is negligible. The ions *m/z* 761 (M-15), *m/z* 671 (M-[15 + 90]) and *m/z* 581 (M-[15 + 2 X 90]) arise out of the loss of a methyl group from the molecular ion and the subsequent loss of one and two derivatised hydroxyl groups, respectively, but all of these fragments are of relatively low intensity. The origin of ion *m/z* 484 (M-292) is unknown, but it could possibly represent the aglycon and a 2-carbon unit of the glucuronide skeleton with a trimethylsilanol group. The loss of 334 mass units, giving rise to the ion *m/z* 442, however, is characteristic of aromatic glucuronides [16], and is formed by cleavage of the glucuronic acid from the molecule and the transfer of a silyl group from the glucuronic acid moiety [16] to the lignan. The ion *m/z* 180 already described is a fragment derived from cleavage of the aromatic ring of the lignan and is the base peak in the mass spectrum of the TMS ether of the unconjugated lignan [1,2,4]. The fragmentation of the glucuronic acid moiety described in [16,17] leads to the occurrence of the typical ions *m/z* 406, *m/z* 407, *m/z* 317 (base peak) and *m/z* 275 which contain the carboxyl group and the ions *m/z* 217 and *m/z* 204. The ion *m/z* 406 is characteristic of aromatic glucuronides representing the loss of a proton from the glucuronic acid moiety. The ion *m/z* 317 is frequently the base peak in the spectra of steroid glucuronide conjugates and the occurrence of these series of ions provides definitive identification of a glucuronide conjugate.

The smaller amounts of the lignan 2,3-bis-(3′-hydroxybenzyl)-butane-1,4-diol present in human urine has made the definitive identification of the glucuronide conjugate from the complete mass spectrum difficult. When computer FIC chromatograms are constructed however, for the above ions, which are characteristic of the Me ester–TMS derivative of glucuronides and the TMS ether derivative of the lignan [4] (fig.2) it can be seen from the relative intensities and from the coincidence in peaking of the ions, that the butanediol (II) is also excreted in urine conjugated to glucuronic acid.

The position of conjugation of the glucuronic acid group remains to be firmly established. On the basis of GC–MS data it was only possible to establish the presence of an aromatic glucuronide, and with two

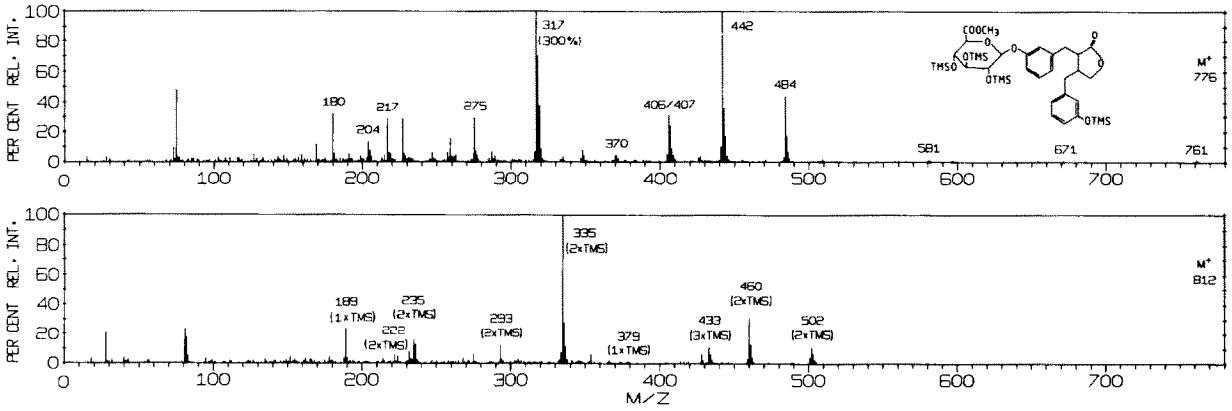

Fig.1. Mass spectra of Me ester–TMS ethers (upper panel) and Me ester–d_9-TMS ethers (lower panel) of 2,3-bis-(3′-hydroxybenzyl)-butyrolactone–glucuronide isolated from urine. The origin of ions is given in the text.

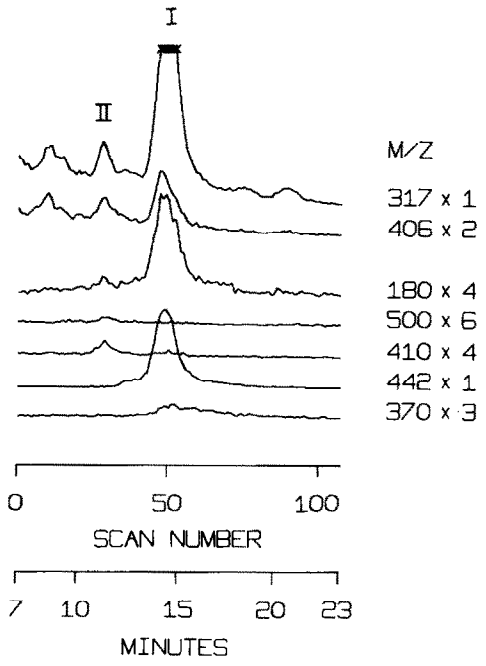

Fig.2. GC–MS analysis of Me ester–TMS ethers of intact lignan glucuronides isolated from urine. FIC chromatograms of *m/z* 317 and *m/z* 406 are representative of the glucuronyl moiety, those of *m/z* 180, *m/z* 500 and *m/z* 410 of the 2,3-bis-(3′-hydroxybenzyl)-butane-1,4-diol structure (II) and those of *m/z* 180, *m/z* 442 and *m/z* 370 of the 2,3-bis-(3′-hydroxybenzyl)-butyrolactone structure (I) (see text).

aromatic rings in the lignan structure, in the case of the butyrolactone the exact position of the glucuronide group could not be ascertained.

Confirmation of the presence of lignan mono- and disulphates was based upon their mobilities on the anion exchange gel TEAP-LH-20 and from their conversion to unconjugated lignans using a mild solvolytic procedure, conditions that will not cleave glucuronide conjugates [8]. Furthermore, when the sulphate fractions were converted to TMS ether derivatives directly without prior solvolysis, TMS ether derivatives of the unconjugated lignans were readily formed. The derivatisation procedure was thus capable of hydrolysing the sulphate conjugate, a reaction which has also been employed for the analysis of other aromatic sulphates [18,19].

3.2. *Quantitative excretion of lignan conjugates in urine*

The total excretion (μg/day) and relative distribution of the two lignans 2,3-bis-(3′-hydroxybenzyl)-butyrolactone (I) and 2,3-bis-(3′-hydroxybenzyl)-butane-1,4-diol (II), between conjugate classes are shown in table 1 for a limited number of normal subjects and one pregnant woman. The relative distribution of these lignans within the conjugate groups was similar for all urine samples with the principal conjugate being a glucuronide. Quantitatively 98% (range 96.0–98.9) of the butyrolactone (I) and 92% (range 87.0–94.7) of the butanediol (II) was excreted as glucuronide conjugates. Similar results have been obtained for the vervet monkey, baboon and rat.

Table 1
Distribution (%) of conjugates of the lignans 2,3-bis-(3′-hydroxybenzyl)-butyrolactone (I) and 2,3-bis-(3′-hydroxybenzyl)-butane-1,4-diol (II) and the total excretion (μg/24 h) in urine from human subjects

Subject	Age	Sex	Unconjugated		Glucuronides		Monosulphates		Disulphates		Total	
			I	II	I	II	I	II	I	II	I	II
NA	6	Male	0.6	0.6	97.9	90.2	1.2	8.6	0.3	0.6	34.0	16.3
KS	25	Male	0.5	0.9	96.3	93.0	2.8	5.6	0.4	0.5	94.3	81.6
AM	12	Female (pre-menarche)	0.2	0.9	98.5	94.6	1.2	4.2	0.2	0.3	66.0	33.3
AT-1	33	Female (cycle day 4)	0.3	<0.1	98.2	92.6	1.3	6.9	0.2	0.6	394.5	17.5
AT-2	33	Female (cycle day 23)	0.2	<0.1	98.4	90.9	1.1	7.8	0.2	1.3	531.1	15.4
BA	62	Female (post-menopausal)	0.1	<0.1	96.0	87.0	3.6	12.3	0.4	0.7	190.9	27.7
BS	28	Female (pregnant 7 weeks)	0.5	1.1	98.9	94.7	0.4	3.2	0.3	1.1	216.2	9.4

Lignans with one sulphate group comprised ~2% (range 0.4–3.6) and 7% (range 3.2–12.3) of the daily excretion of I and II, respectively. Only small amounts of lignans were identified as disulphates (0.2–1.3%). In spite of the precautions taken during collection of the urine it is possible that the small amounts of unconjugated lignans (0–1.1%) detected in urine resulted from the hydrolysis of the lignan conjugates.

In general, though the mode of conjugation of the lignans resembled that of steroid hormone metabolites [20] and although not proven yet, their conjugation probably occurs in the liver.

The daily excretion of 2,3-bis-(3′-hydroxybenzyl)-butyrolactone (I) appeared to vary with age and sex (table 1). The highest levels have been found in fertile women. The excretion of 2,3-bis-(3′-hydroxybenzyl)-butane-1,4-diol (II) appeared to be less variable and thus ratios between I and II were found to be much higher for fertile women, including the pregnant women (about 23–34) compared with men and non-fertile women (about 1–7). The presence of these two lignans in the urine from children is confirmed from the FIC chromatograms shown in fig.3, although the quantitative excretion of the two lignans in children has been found to be lower than in adults, and

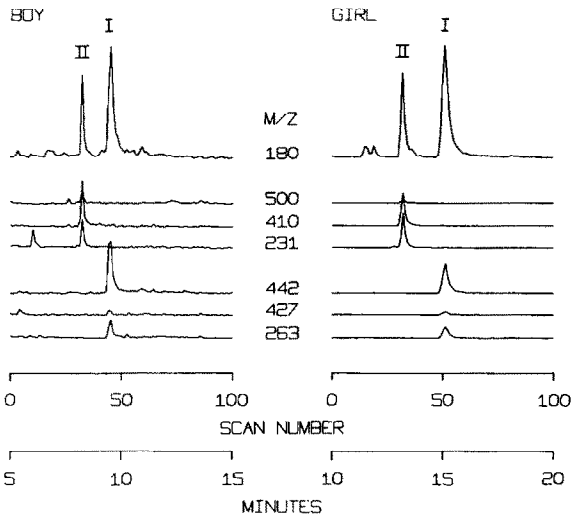

Fig.3. GC–MS analyses of lignans in hydrolysed glucuronide fractions from the urine from a boy (16 years) and a girl (12 years). FIC chromatograms were constructed by the computer for characteristic ions given by TMS ethers of: 2,3-bis-(3′-hydroxybenzyl)-butane-1,4-diol (II), *m/z* 180, *m/z* 500, *m/z* 410 and *m/z* 231; and 2,3-bis-(3′-hydroxybenzyl)-butyrolactone (I), *m/z* 180, *m/z* 442 (M^+), *m/z* 427 and *m/z* 263.

almost undetectable in the first days of life [1,3].

The number of urine samples analysed in this study is small, and since the origin, metabolism and the physiological role of these new lignans is presently unknown, the significance of these results remains to be seen.

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References

[1] Setchell, K. D. R., Lawson, A. M., Mitchell, F. L., Adlercreutz, H., Kirk, D. N. and Axelson, M. (1980) Nature 287, 740.
[2] Setchell, K. D. R., Lawson, A. M., Conway, E., Kirk, D. N., Cooley, G., Farrant, R. D. and Axelson, M. (1980) submitted.
[3] Setchell, K. D. R., Lawson, A. M., Axelson, M. and Adlercreutz, H. (1980) Res. Steroid 9, 207.
[4] Setchell, K. D. R., Bull, R. and Adlercreutz, H. (1980) J. Steroid Biochem. 12, 375.
[5] Axelson, M., Sahlberg, B.-L. and Sjövall, J. (1980) submitted.
[6] Few, J. D. (1961) J. Endocrinol. 22, 31.
[7] Tserng, K.-Y. and Klein, P. D. (1977) Steroids 29, 635.
[8] Jacobsohn, G. M. and Lieberman, S. (1962) J. Biol. Chem. 237, 1469.
[9] Rutten, G. A. F. M. and Luyten, J. A. (1972) J. Chromatogr. 74, 177.
[10] Van den Berg, P. M. J. and Cox, T. P. H. (1972) Chromatographia 5, 301.
[11] Reimendal, R. and Sjövall, J. (1972) Anal. Chem. 44, 21.
[12] Axelson, M. and Sjövall, J. (1977) J. Steroid Biochem. 8, 683.
[13] Axelson, M., Cronholm, T., Curstedt, T., Reimendal, R. and Sjövall, J. (1974) Chromatographia 7, 502.
[14] Almé, B., Norden, Å. and Sjövall, J. (1978) Clin. Chim. Acta 86, 251.
[15] Almé, B. and Sjövall, J. (1980) J. Steroid Biochem. 13, 907.
[16] Spiegelhalder, B., Röhle, G., Siekmann, L. and Breuer, H. (1976) J. Steroid Biochem. 7, 749.
[17] Billets, S., Lietman, P. S. and Fenselau, C. (1973) J. Med. Chem. 16, 30.
[18] Setchell, K. D. R., Cummings, A. J. and Lawson, A. M. (1978) in: Quantitative mass spectrometry in life sciences II (De Leenheer, A. P. et al. eds) pp. 263–273, Elsevier/North-Holland, Amsterdam, New York.
[19] Murray, S,, Baillie, T. A. and Davies, D. S. (1977) J. Chromatogr. 143, 541.
[20] Setchell, K. D. R., Almé, B., Axelson, M. and Sjövall, J. (1976) J. Steroid Biochem. 7, 615.