Identification and quantitative estimation of a lignan in human and bovine semen

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Summary. The lignan identified in human and bovine semen was *trans* 2,3-bis(3'-hydroxybenzyl)- γ -butyrolactone. It was present in unconjugated and conjugated forms. Mean seminal plasma concentrations were always higher than the corresponding blood plasma levels (between 2.5 and 25 times higher), indicating an efficient clearance capacity of the male reproductive system.

Introduction

The occurrence of lignans, mainly *trans* 2,3-bis(3'-hydroxybenzyl)- γ -butyrolactone (enterolactone, Text-fig. 1), in urine, bile, faeces and plasma of human and other animal species (monkey, rat) has been extensively discussed (Setchell, Bull & Adlercreutz, 1980; Setchell, Lawson, Axelson & Adlercreutz, 1980; Setchell *et al.*, 1981 a, b). Simultaneously, Stitch *et al.* (1980) have shown enterolactone to have characteristic excretion patterns in ovulatory cycles and in pregnancy. Conjugation of lignans, mainly as glucuronides (Axelson & Setchell, 1980), and evidence for an intestinal bacterial origin (Axelson & Setchell, 1981; Setchell *et al.*, 1981 c) have been reported.

We have investigated the concentrations of enterolactone in seminal fluid of men and bulls.

Text-fig. 1. The structure of *trans* 2,3-bis(3'-hydroxybenzyl)- γ -butyrolactone (enterolactone).

Materials and Methods

Blood and semen collection

Ejaculates from 6 men were collected by masturbation after at least 3 days of abstinence. They were qualitatively and quantitatively evaluated by the criteria given by Jondet & Tea (1981) and proven to have normal spermograms. Peripheral blood samples were taken the same day into vacuum tubes containing EDTA.

Four Montbéliarde bulls of about 1 year of age, fed for the previous 3 months on hay (10 kg/day) and concentrates (4 kg/day), were allowed to ejaculate weekly during 1 month. Ejaculates were collected into an artificial vagina and evaluated according to classical criteria,

described previously by Thibier & Colchen-Bourlaud (1972). Peripheral blood was withdrawn by jugular venepuncture into heparinized vacuum tubes.

All blood and semen samples were centrifuged (3000 g, 10 min) and the corresponding plasmas were stored at -20°C until analysis.

Synthetic enterolactone

Enterolactone was synthesized according to Groen & Leemhuis (1980), Cooley, Farrant, Kirk & Wynn (1981) and Pelter, Satyanarayana & Ward (1981). Pure reference compound was generously provided by Professor D. N. Kirk (Westfield College, London, U.K.) and by Dr M. B. Groen (Scientific Development Group, Organon B.V., Oss, The Netherlands).

Deuterium labelled enterolactone

Enterolactone was labelled by an exchange reaction similar to that described for oestrogens (Dehennin, Reiffsteck & Scholler, 1980). Isotope composition measurements indicated a d_0/d_4 ratio of 0.9%. A stock solution of [$^2\text{H}_4$]enterolactone in pyridine (made anhydrous by refluxing on potassium hydroxide and fractional distillation) was prepared and the exact concentration was determined by gas chromatography. The stability of the labelled analogue was checked and proved to be sufficient for use as an internal standard.

Synthetic enterolactone and human sperm motility

A 5 μl sample of a 1 $\mu\text{mol/l}$ solution of enterolactone in phosphate saline buffer (0.1 M, pH 7.4) was added to 5 μl liquefied human semen. The percentage motile spermatozoa and sperm velocity were determined immediately, and at 1 and 2 h after mixing on a microscope slide.

Extraction, purification and derivatization

An amount of [$^2\text{H}_4$]enterolactone (in 20 μl ethanol) equivalent to the amount of natural compound was added to 1 ml of blood or seminal plasma. After 30 min for equilibration, the sample was applied to an extraction column containing 1 g (7×1 cm) Extrelut (Merck, Darmstadt, West Germany) and soaked for 20 min. Elution with 5 ml diethyl ether gave, after evaporation, an extract which was applied to a column (5×0.4 cm) of Sephadex LH-20 (Pharmacia Fine Chemicals, Uppsala, Sweden) swollen in benzene-ethanol (85:15, v/v). The first 3 ml of eluant were discarded and enterolactone was eluted in the next 2.5 ml. Evaporation of the solvent and derivatization with *N,O*-bis (trimethylsilyl)-trifluoroacetamide containing 5% trimethylchlorosilane, in dry pyridine, yielded the corresponding trimethylsilyl ethers (TMS).

An enzymic hydrolysis step with *Helix pomatia* digestive juice was included, prior to the addition of [$^2\text{H}_4$]enterolactone, when total enterolactone concentrations were determined. Blood or seminal plasma samples (1 ml) were diluted up to 10 ml with acetate buffer (0.2 M, pH = 5.2) and incubated overnight at 37°C with 10^3 β -glucuronidase units and 10^4 sulphatase units. The mixture was brought to neutrality, diluted up to 20 ml with water and extracted with 20 ml diethyl ether after soaking on a column containing 10 g Extrelut (6×3 cm). Further treatment was identical to that described for unconjugated enterolactone.

Gas chromatography-mass spectrometry (GC-MS)

GC-MS was performed on a glass capillary column (25×0.3 mm) deactivated by persilylation and statically coated with OV-73 stationary phase. The column, fitted to an all-glass solid injection system, was operated at 230°C under 10^5 Pa helium pressure and

directly coupled to a quadrupole mass spectrometer (Nermag, Rueil-Malmaison, France). Electron impact spectra were obtained under normal operating conditions and selected ion monitoring was performed on the molecular ions (M^+) of enterolactone-TMS (M^+ , 442) and [2H_4]enterolactone-TMS (M^+ , 446).

Quantitative analysis

Quantitative determinations were made according to the isotope dilution principle outlined previously (Dehennin, Reiffsteck, Scholler, Cohen & Bizollon, 1980). Intra-assay variability was assessed on duplicate measurements which had relative standard deviations between 3 and 4%.

Results

The identification of enterolactone in seminal fluid was based on the comparison of the mass spectra of underivatized and seminal compound, which revealed complete identity with prominent peaks at mass-to-charge ratios (m/z) of 298 (M^+ , 8.0%), 191 (7.9%), 133 (15.2%), 107 (100%) and 106 (54.5%). The same close similarity was observed for the TMS derivative with characteristic peaks at m/z 442 (M^+ , 13.2%), 263 (6.6%), 180 (100%), and 73 (84.2%). A second identification criterion was the nearly identical chromatographic behaviour of enterolactone-TMS and its deuterated isotopomer which had, as could be expected for deuterium-labelled compounds, a slightly shorter retention index ($I_{230}^{OV-73} = 2845$ for enterolactone-TMS and 2842 for [2H_4]enterolactone-TMS).

Seminal plasma and corresponding blood plasma levels are shown in Table 1. Total enterolactone concentrations should be considered less accurate than the unconjugated ones owing to possible incompleteness of the enzymic hydrolysis which could not be corrected by the use of an unconjugated labelled analogue.

Table 1. Concentrations (nmol/l) of enterolactone in seminal and blood plasma of normal men and bulls

		Men (N = 6)		Bulls (N = 4)	
Enterolactone		Mean	Range	Mean	Range
Semen	Unconjugated	43	8.0–127	604	560–651
	Total	183	63–557	3200	3100–3300
Blood	Unconjugated	14.3	10.3–21	24	21–27
	Total	73	37–127	228	198–258

There were no changes in the percentage motile spermatozoa or sperm motility of human ejaculates mixed with 1 μ mol/l concentrations of synthetic enterolactone.

No immunological cross-reactivity could be assessed with an antiserum raised against oestrone-17-carboxymethyl-BSA conjugate in the rabbit and which is currently used for oestradiol-17 β determinations by radioimmunoassay (Castanier & Scholler, 1970).

Attempts to identify a second lignan, 2,3-bis(3'-hydroxybenzyl)-butane-1,4-diol, were unsuccessful.

Discussion

Enterolactone was detected during our investigations on the identification and the quantitative determination of seminal oestrogens by isotope dilution-mass spectrometry (Reiffsteck,

Dehennin & Scholler, 1981). The aromatic structure confers on enterolactone some chromatographic properties similar to those of oestrogens. No oestrogen-like action on mouse uterine weight could however be detected with enterolactone doses up to 67 $\mu\text{mol/kg}$ body weight (Setchell *et al.*, 1981c).

Bacteriological studies have suggested that *Clostridia* from intestinal microflora may be the principal source of enterolactone in man (Setchell *et al.*, 1981c). According to Adlercreutz *et al.* (1981) and M. B. Groen (personal communication), enterolactone would be a metabolite of lignin from the fibrous constituents of the diet. These suggestions are corroborated by the high enterolactone levels and the limited concentration ranges found in bovine semen and blood. Indeed, cattle daily consume large amounts of lignin present in grass or forage (Harkin, 1973) and the rumen contains huge numbers (10^5 – 10^6 per ml rumen content) of *Clostridium lochheadii* and *Clostridium longisporum* (Hungate, 1966). It should also be taken into account that the bovine semen and blood concentration ranges of enterolactone are related here to their strictly similar diet.

In human semen, and to a lesser degree also in blood, enterolactone levels were considerably lower. There was a high inter-individual variability which can, to some extent, be related to the variety of the human diet.

The ratio of unconjugated to conjugated enterolactone from semen and blood is largely in favour of the latter, but the proportion of unconjugated compound is significantly higher than that which has been observed in urine of the human male (Axelson & Setchell, 1980).

It therefore seems, if the exogenous origin is confirmed, that enterolactone readily passes into the blood stream by enterohepatic circulation (Axelson & Setchell, 1981; Setchell *et al.*, 1981c). Eiler & Graves (1977, 1981) and Graves & Eiler (1979) have demonstrated the possibility of a rapid transfer of exogenous oestradiol-17 β , cortisol, testosterone and progesterone from the bovine circulatory system to the accessory sex glands and to semen at ejaculation. The clearance capacity of the male reproductive system might be a plausible explanation for the occurrence of enterolactone in semen.

Furthermore, high concentrations of enterolactone, such as those found in cattle, do not seem to be detrimental to semen output and qualitative traits, or to fertility. The bulls from which samples were collected were quite representative of the population with average semen quality and conception rate, similar to those reported by Abdel Malak & Thibier (1982). However, this lignan could affect fertility after passing to the female genital tract during coitus, although possible physiological implications of enterolactone seem controversial. Many plant lignans are known to have antimutagenic activity (Hartwell, 1976), while some clostridia are able to synthesize aromatic compounds which might be potentially carcinogenic (Goddard & Hill, 1972).

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