The identification of the weak oestrogen equol [7-hydroxy-3-(4'-hydroxyphenyl)chroman] in human urine

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The identification (by gas chromatography–mass spectrometry and n.m.r.) for the first
time of the weak oestrogen equol [7-hydroxy-3-(4'-hydroxyphenyl)chroman] in human
urine is described. Preliminary results of its quantitative excretion in urine are reported
and the potential significance of the occurrence of this compound is discussed.

Equol [7-hydroxy-3-(4'-hydroxyphenyl)chroman]
is a heterocyclic phenol (Fig. 1) that was first
isolated and identified from pregnant-mares' urine
(Marrian & Haslewood, 1932) and later found in
the urine of the goat (Klyne & Wright, 1957), cow
(Klyne & Wright, 1959), hen (MacRae et al., 1960;
Common & Ainsworth, 1961) and sheep (Braden et
al., 1967; Shutt & Braden, 1968). It has been shown
to possess weak oestrogenic activity (Shutt &
Braden, 1968; Shutt & Cox, 1972), bind to uterine
receptor sites (Shutt & Cox, 1972) and account for
an infertility syndrome in sheep (Bennetts et al.,
1946; Moule et al., 1963; Morley et al., 1964).

We report here structural-elucidation studies
using gas chromatography–mass spectrometry and
n.m.r. spectrometry, which establish for the first time
the presence of equol in human and rat urine.

Materials and methods

Urine collections

Urine collections (24 h) were obtained from
normal men and women (aged 6–62 years) and from
mature rats of the Sprague–Dawley strain. The urine
was collected in glass containers with no preser-
vative added, frozen immediately and stored at
−20°C until required for analysis.

Extraction and isolation of equol for gas chromato-
graphy–mass spectrometry analysis

Equol was extracted from urine by using the
neutral resin Amberlite XAD-2 and the individual
conjugated forms isolated by column chromato-
graphy on the anion-exchange gel triethylamino-
hydroxypropyl-Sephadex LH-20 (TEAP-LH-20) as
described in detail previously (Axelson & Setchell,

1980, 1981). Low- and high-resolution gas
chromatography–mass spectrometry analyses of the
trimethylsilyl ether derivative were carried out after
hydrolysis and solvolysis of the conjugated fractions
(Axelson & Setchell, 1980, 1981).

Preparation of trimethylsilyl ether and ^2H-labelled
trimethylsilyl ether derivatives

Trimethylsilyl ethers were prepared by addition of
100 μl of a solution of pyridine/hexamethyldisilaz-
ane/trimethylchlorosilane (3:2:1, by vol.) and heat-
ing at 60°C for 1 h. The silylation reagents were
removed under a stream of nitrogen and the deriva-
tives redissolved in hexane.

$^2H_9$-labelled trimethylsilyl ethers were prepared by
addition of 100 μl of [$^2H_9$]trimethylchlorosilane/
pyridine (20:1, v/v) under the same conditions.

Gas chromatography

Gas chromatography was performed on either a
Pye 106 gas chromatograph equipped with flame-
ionization detectors and modified to accept a
25 metre wall-coated open tubular-glass capillary
column coated with silicone OV-1 (Jaeggi, Trogen,
Switzerland), or a modified Pye 104 gas chromato-
graph housing a 25-metre open tubular-glass capil-
lary column coated with SE-30 (Rutten & Luyten,
1972). Helium was used as the carrier gas, with a
flow rate through the column of 1 ml/min. Samples
were applied to the column via an all-glass solid-
injection system of the type described by Van den
Berg & Cox (1972). Analyses were performed by
using: (i) temperature-programmed operation from
175° to 275°C with increments of 2°C/min; and (ii)
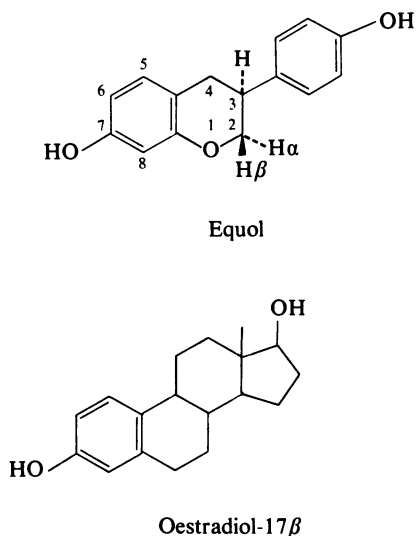isothermal operation at 260°C.

Fig. 1. *The chemical structure of equol compared with that of oestradiol-17β*

Gas chromatography–mass spectrometry

Gas chromatography–mass spectrometry with high and low resolution was carried out by using the following instruments.

(i) A Varian MAT-731 double-focusing mass spectrometer coupled to either a Varian 2700 gas chromatograph housing a conventional glass column (2 m × 4 mm int.diam.) packed with silicone OV-1 coated on Chromasorb W or a Pye 106 gas chromatograph housing a silicone OV-1 glass capillary column connected to the ion source via an open coupling (Henneberg *et al.*, 1975). Low-resolution mass spectrometry was carried out with either single or repetitive magnetic scanning over the range 0–1000 atomic mass units (a.m.u.) under the following conditions: temperature of transfer line, 250°C; accelerating voltage, 8 kV; ionization current 800 μA; ionization voltage 70 eV. A resolving power of 10 000 was used for accurate mass measurements.

(ii) A modified LKB 9000 having an open tubular-glass capillary column (25 m × 0.3 mm) coated with SE-30 and connected to the ion source via a single-stage adjustable jet separator (Reimendal & Sjövall, 1972; Axelson & Sjövall, 1977). The temperatures of the column, molecular separator and ion source were 250, 275 and 290°C respectively. The energy of the bombarding electrons was 22.5 eV, the ionization current was 60 μA, and the accelerating voltage was 3.5 kV. Repetitive magnetic scanning (usually 10 scans/min) over the mass range *m/z* 0–800 a.m.u. was initiated after a suitable delay after injection of the sample. Methods for the

computerized recording and evaluation of mass-spectral data have been described elsewhere (Axelson *et al.*, 1974).

Isolation of equol for analysis by n.m.r. spectrometry

The sample of equol analysed by n.m.r. spectrometry was obtained after direct enzymic hydrolysis of urine extracts and isolation of the liberated unconjugated compounds by column chromatography on columns of TEAP-LH-20 (Axelson & Setchell, 1981). Diphenolic compounds were then separated from monophenolic compounds on small columns of DEAE-Sephadex (Pharmacia, Uppsala, Sweden) prepared in 72% (v/v) methanol in the OH⁻ form (Axelson & Setchell, 1981). The diphenolic fraction, eluted by 72% methanol saturated with CO_2, was isolated after first recovering mono-phenolic compounds by elution with 72% methanol, and was found by analytical t.l.c. [Merck pre-coated silica-gel 60 F-254 plates run in ethyl acetate/cyclohexane (2:1, v/v)], to contain at least five components, of which equol was the least polar ($R_F \sim 0.6$). Equol was isolated from this extract by preparative t.l.c. [20 × 20 cm plates with a 1 mm layer of Merck Kielselgel 60 G/Kieselgel 60 PF 254 + 366 (1:1, w/w) run in the same solvent] scraped from the plate and then eluted with ethanol to give a sample of 0.7 mg, which was used for n.m.r. spectroscopy without further purification.

N.m.r. spectrometry

N.m.r. spectra (at 100 MHz) were recorded on a Jeol FX100 instrument (kindly provided by the Medical Research Council for the Steroid Reference Collection). Spectra [chemical shift (δ) (p.p.m.)] were determined in [²H]chloroform relative to internal tetramethylsilane. The 400 MHz spectrum was kindly provided for the same solution by Dr. G. E. Hawkes, Queen Mary College, University of London, with a Bruker WH-400 spectrometer.

Results and discussion

During the course of our recent studies on lignans (Setchell *et al.*, 1980, 1981), equol was described as an unidentified phenolic compound in human and rat urine (Axelson & Setchell, 1980).

From its chromatographic mobilities on the strong and weak anion-exchange gels (TEAP-LH-20 and DEAE-Sephadex respectively) it was shown to possess two aromatic hydroxy groups (Axelson & Setchell, 1980; Axelson & Setchell, 1981), and because it was relatively stable in an alkaline environment, a catechol structure was excluded. It appeared to have no reactive carbonyl groups, since reduction by lithium aluminium hydride (Setchell *et al.*, 1981) or formation of *O*-methyl oximes (Thenot
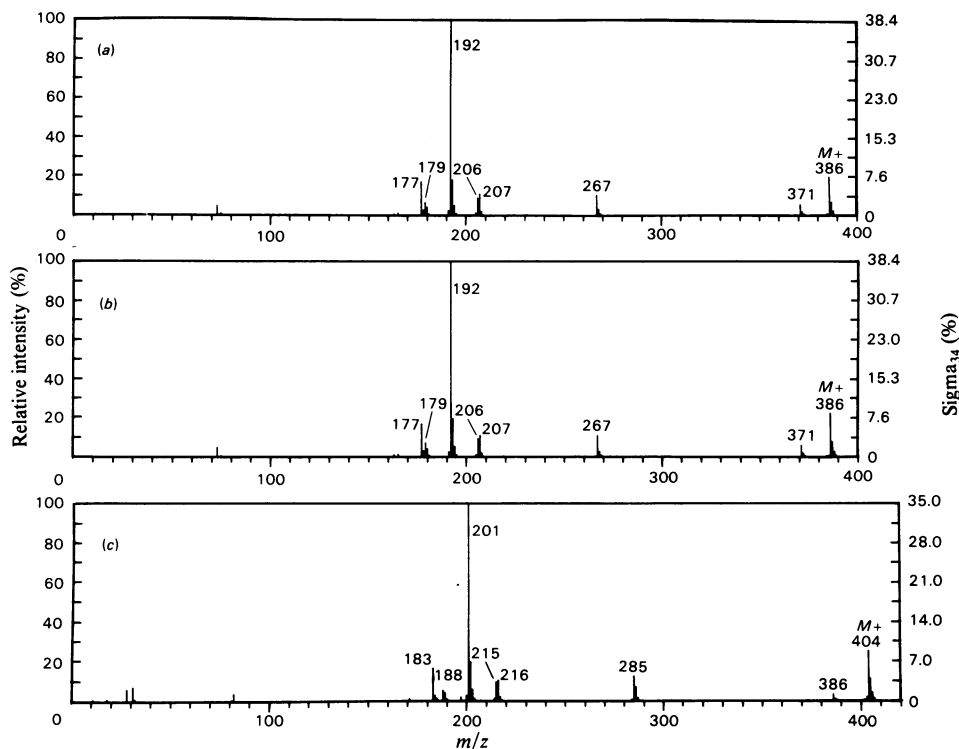
Fig. 2. *Electron-impact-ionization (22.5 eV) mass spectra of the trimethylsilyl (TMS) ether derivative of (a) an authentic sample of equol, and (b) equol isolated from human urine*

The mass spectrum (*c*) of the ^{2}H-labelled trimethylsilyl ether derivative of equol isolated from human urine is also presented. Sigma$_{34}$(%) is a measure of the proportion of ionization carried by each ion above mass m/z 34.

& Horning, 1972), were unsuccessful. Definitive evidence that this diphenolic compound was equol, however, was obtained after its analysis by gas chromatography–mass spectrometry and n.m.r. spectrometry and comparison with the authentic compound.

Gas chromatography–mass spectrometry

To our knowledge, a detailed mass-spectrometric analysis of equol has never been described, although a list of the major ions of the trimethylsilyl ether derivative (Carignan *et al.*, 1978) and of the free compound (Hayashi *et al.*, 1978) have been reported previously. The mass spectra (Fig. 2) and gas-chromatographic retention time (25.30 Methylene Units) of the trimethylsilyl ether derivative of this diphenolic compound isolated from urine and an authentic sample of equol (obtained from the MRC Steroid Reference Collection, Westfield College, University of London) were found to be identical. Some aspects of the fragmentation pattern have been discussed previously (Axelson & Setchell, 1981).

The molecular ion at m/z 386 is intense, owing to stabilization of the positive charge by the aromatic system. Evidence for two derivatizable hydroxy groups in the molecule was obtained from the mass increase (18 mass units) in the [^{2}H$_9$]trimethylsilyl ether derivative (Fig. 2). Accurate mass determination of the molecular ion indicated its composition as $C_{21}H_{30}O_3Si_2$ (386.1730 a.m.u.). The ion of m/z 371 (*M*-15) is formed by loss of a methyl group from one of the trimethylsilyl groups. The mechanism of formation of the ion m/z 267 is uncertain, but this fragment contains two trimethylsilyl ether groups, evidenced from the [^{2}H$_9$]trimethylsilyl derivative (Fig. 2). The high-resolution analysis shows its composition to be $C_{13}H_{23}O_2Si_2$ (267.1241 a.m.u.), consistent with a tropylium ion substituted with two trimethylsilanoxy groups, and in equol is probably formed by a cleavage of the ether linkage (O–C_2) and the carbon bridge (C_3–C_4) with subsequent transfer of a silyl group to the aromatic oxygen atom. A similar rearrangement process has been reported for aromatic glucuronides

(Billets et al., 1973; Spielgelhalder et al., 1976). The ion at m/z 207 is formed from the molecular ion by loss of C_7H_7-$OSi(CH_3)_3$, a fragment which with retention of charge is found at m/z 179. This ion is analogous to the substituted tropylium ions postulated in spectra of underivatized isoflavans (Pelter et al., 1965). A further similarity with these spectra is the retro Diels–Alder rearrangement of the pyran ring of equol to give the base peak at m/z 192 with the expected atomic composition for $(CH_3)_3SiO$-C_6H_4-CH=CH_2.

N.m.r. analysis

N.m.r. spectra do not appear to have been reported previously for equol. The following assignments assume that the p-hydroxyphenyl substituent has the equatorial conformation at C-3. The ^1H spectrum of equol isolated from urine showed superimposed multiplets (at $100\,MH_z$) in the region 2.8–3.2 p.p.m. (3-H and 4-H_2), a triplet at 3.91 p.p.m. (2β-H; axial) and a double doublet at 4.22 (2α-H; equatorial). The protons at C-2 and C-3 comprise an ABM three-spin system, with $J_{2\alpha,2\beta} \approx 10.5\,Hz$, $J_{2\beta,3} \approx 9.5\,Hz$ and $J_{2\alpha,3} \approx 3\,Hz$, from an analysis of the 'AB' part of the spectrum. The 'M' proton (3-H) is further coupled to the protons at C-4, with second-order effects giving rise to the complex multiplets near 3 p.p.m. The spectrum at 400 MHz separated these signals into a very close pair of apparent singlets (probably strongly perturbed doublets) at 2.91 and 2.93 p.p.m. (4-H_2) and a complex multiplet comprising at least ten components between 3.12 and 3.20 p.p.m. (3-H).

The aromatic part of the 100 MHz spectrum showed a pair of doublets at 6.82 and 7.09 p.p.m. (protons of p-hydroxyphenyl group; $J_{ortho} = 8.5\,Hz$), a doublet at 6.87 p.p.m. (5-H; $J_{ortho} = 8\,Hz$), a double doublet at 6.38 p.p.m. (6-H; $J_{ortho} = 8\,Hz$, $J_{meta} = 2.5\,Hz$), and a superimposed singlet at 6.35 p.p.m. (8-H). The signals due to 6-H and 8-H were just separated at 400 MHz, when the signal at 6.35 p.p.m., freed from second-order effects, appeared as a doublet ($J_{meta} = 2.5\,Hz$). The ^1H n.m.r. spectrum of an authentic sample of equol was identical with that of the compound isolated from urine.

Quantitative excretion and potential physiological significance of equol to man

Equol belongs to a class of compounds called isoflavans and was first isolated by Marrian & Haslewood from pregnant-mares' urine as long ago as 1932 (Marrian & Haslewood, 1932). It was originally considered to possess no oestrogenic activity (Marrian & Haslewood, 1932), but on closer examination was later found to be weakly oestrogenic, possessing 10^{-3}–10^{-5} times the activity of oestradiol-17β (Shutt & Braden, 1968; Shutt & Cox, 1972) to which it bears some structural similarity

(Fig. 1) More recently, equol was shown to be antagonistic to oestradiol-17β by competing for cytoplasmic oestrogen receptors (Tang & Adams, 1980).

Interest in equol and other isoflavanoids was stimulated after they were incriminated in the infertility syndrome of sheep, first described in Western Australia (Bennetts et al., 1946), in which a cystic condition accompanied by a failure to conceive occurred in animals grazing on certain species of clover (Bennetts et al., 1946; Moule et al., 1963; Morley et al., 1964). Subsequent analysis of clover leaves revealed the presence of a number of phyto-oestrogens of isoflavanoid structure (Bradbury & White, 1954), and extensive investigation by several groups of workers of the metabolism by rumen contents of one of these, formononetin, confirmed that it was demethylated and reduced by microflora to give equol (Shutt & Braden, 1968; Batterham et al., 1965; Nilsson et al., 1967; Batterham et al., 1971). Equol is readily absorbed from the gastrointestinal tract and conjugated to glucuronic acid in the liver (Shutt et al., 1970; Lindner, 1967) and high plasma concentrations of equol are now recognized as responsible for the infertility syndrome named 'clover disease' in sheep (Shutt et al., 1970; Lindner, 1967).

Although equol has also been identified in the urine of the goat (Klyne & Wright, 1957), cow (Klyne & Wright, 1959), hen (MacRae et al., 1960; Common & Ainsworth, 1961) and guinea pig (Morley et al., 1968), its occurrence in humans has to our knowledge never been previously described.

The excretion of equol in the urine of six normal humans was found to be quantitatively similar to the classical oestrogens (although it was not related to hormonal status), and equol was shown to be excreted almost exclusively as the monoglucuronide conjugate (Table 1). These observations are consistent with our previous studies of the rat (Axelson & Setchell, 1981) in which equol was also shown to exhibit an enterohepatic circulation and to be present in high concentrations in bile and portal-venous blood (Axelson & Setchell, 1981).

As demonstrated previously in animals (Shutt, 1976; Axelson & Setchell, 1981) equol in man is probably formed by the action of gut microflora on an isoflavanoid plant precursor(s) and is not derived from the ingestion of equol in food. In our recent studies we have found that soya food contains a high content of precursor(s) that can be converted into equol (M. Axelson, J. Sjövall, B. E. Gustafsson & K. D. R. Setchell, unpublished work), and that with the increasing use of soya beans as a protein food source, this may be a major dietary source of equol in man.

The significance to humans of the presence of a weak oestrogen-like equol remains to be evaluated.

Table 1. *Distribution (%) of conjugates of equol and their total excretion (μg/24 h) in urine from humans and rats*

Subject	Age	Details	Distribution (%)			Total excretion (μg/day)
			Glucuronide	Monosulphate	Disulphate	
1	6	Male	99.9	<0.1	<0.1	35.2
2	25	Male	99.9	<0.1	<0.1	10.9
3	12	Female (premenarche)	99.8	<0.1	<0.1	43.3
4a	33	Female (follicular phase)	94.3	4.3	1.4	13.2
4b	33	Female (luteal phase)	90.1	4.0	5.9	10.7
5	62	Female (postmenopausal)	99.9	<0.1	<0.1	28.3
Rat	—	Male	99.7	0.1	0.2	128.6
Rat	—	Female	99.9	0.1	0.1	50.0

The marked contraceptive effect of equol on the reproductive capability of animals after ingestion of plants rich in phyto-oestrogens or their precursors, leads us to consider whether there may be some value in quantitatively screening for plant oestrogens in women with clinically and biochemically unexplained infertility or menstrual-cycle disorders.

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