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Sex differences in the conjugation of tiaramide by the CR/CD and JCL/SD strains of albino rat

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The metabolism of tiaramide (Sotine, Solantal) (Fig. 1) in a number of species including the male JCL/SD strain of rat has been reported previously [1, 2]. Metabolites of tiaramide excreted in the urine of male rats are formed mainly by *N*-dealkylation, *N*-oxidation or oxidation of a hydroxyethyl grouping to a carboxylic acid function. Conjugation reactions, principally glucuronidation, form a very minor pathway (< 1%). Recently, we have investigated the metabolism of tiaramide by male and female adult CR/CD and female JCL/SD strain rats both after oral and intravenous administration of 100 mg/kg tiaramide. The drug was administered as its hydrochloride salt. The metabolic products excreted in the urine of the male CR/CD rat were the same as those excreted in the urine of the male JCL/SD strain of rat previously reported. The female CR/CD rat in contrast excreted in the urine a significant proportion of the dose (20%) as a previously undetected metabolite. This metabolite was also the major excretory product detected in the urine of female JCL/SD strain rats. This metabolite was not detectable in the urine of male rats of either strain. Analysis of bile collected from anaesthetised female CR/CD strain rats receiving 50 mg/kg tiaramide intravenously indicated that biliary excretion of the unknown metabolite represented 35–45% of the administered dose. The metabolite was also present in the bile of male CR/CD strain rats receiving a similar dose but represented only 3–5% of the administered dose. The remaining metabolites in the urine from both strains of female rat were the same as for male rats. The route of administration had no effect on the results. The identity of the unknown metabolite was investigated.

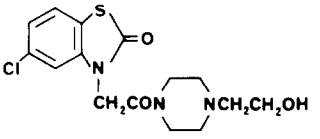

Fig. 1. Structure of tiaramide.

Urine was collected for 24 hr from female CR/CD and JCL/SD strain rats following an oral dose (100 mg/kg) of tiaramide. The urine was adjusted to pH 10 with 1 M NaOH and applied to a column (10 × 1 cm i.d.) packed with Amberlite XAD-2 resin. After washing the resin bed with 30 ml of water, 60 ml of methanol was passed through the column. The methanolic eluate was dried *in vacuo* at a temperature not exceeding 60°, redissolved in a small volume of methanol and chromatographed on a 2 mm silica gel $60F_{254}$ preparative layer chromatography plate (Merck, Darmstadt, F.R.G.) developed in acetone: 0.88 sp. g ammonia (25:1 v/v). The silica gel corresponding to the metabolite was removed from the plate and eluted with methanol. The eluate was evaporated to dryness under reduced pressure and the chromatography and elution repeated as above. The isolated metabolite was triturated twice with a small volume of petroleum ether (40–60° b.p.) and dried. The metabolite was analysed by mass spectrometry on a Kratos MS 50 mass spectrophotometer linked to a DS 50S data system. The sample was introduced by a direct insertion probe. No molecular ion was detected but principal ions were present at *m/e* values of 337, 324, 226, 198 and 170. The mass spectrum of the metabolite was consistent with a conjugate of tiaramide in which conjugation had occurred on the hydroxyethyl chain. The loss of the conjugating moiety could be envisaged to produce the ion at *m/e* 337 in the spectrum.

Acid hydrolysis of the metabolite yielded a material identical to tiaramide. The metabolite was not hydrolysed following treatment with 500 units of β-glucuronidase (Bacterial type 1, Sigma Chemical Co., Poole, U.K.) at 37° for 24 hr in 0.1 M phosphate buffer, pH 6.8, and therefore the possibility that the material was the sulphate conjugate of tiaramide was investigated.

Male and female CR/CD rats were administered two intraperitoneal doses of sodium [^{35}S]sulphate (100 μCi) 24 and 0.5 hr before a single oral dose of tiaramide (50 mg/kg). Urine samples were collected after each administration of sodium [^{35}S]sulphate. Urine samples were extracted as above with Amberlite XAD-2 resin and chromatographed in chloroform: methanol: diethylamine (15:4:1, v/v) and acetone: 0.88 sp. g ammonia (25:1 v/v). Extracts of male and female rat urine collected before tiaramide dosing did not contain any detectable radiolabelled material co-chromatographing with the unknown metabolite. The extracts of urine collected after tiaramide dosing contained a ^{35}S-radiolabelled band demonstrated by autoradiography to co-chromatograph with the unknown metabolite. This band was intense in the extracts of urine from female rats but not discernable in the extracts of male rat urine. The incorporation of ^{35}S-sulphate into the metabolite suggested that it was the sulphate conjugate of tiaramide. Interestingly, there was no detectable hydrolysis when the metabolite was incubated at 37° for periods of up to 72 hr with 1200 units of *Helix pomatia* aryl sulphatase (Sigma) in 0.1 M sodium acetate buffer (pH 5.0). Confirmation that the metabolite was the sulphate conjugate of tiaramide was achieved by chemical synthesis. Tiaramide hydrochloride (500 mg) was dissolved in 2 ml of concentrated sulphuric acid and allowed to react under ambient conditions for 3 hr. An identical product was produced by reacting tiaramide hydrochloride with an equimolar proportion of chlorosulphonic acid dissolved in dioxan (1:5, v/v) under similar conditions. The synthetic product and the isolated metabolite appeared identical by thin-layer chromatography on 0.5 mm silica gel 60 F_{254} TLC plates (Merck) using ethanol, butan-1-ol: water (18:2, v/v), ethyl acetate: acetic acid: water (14:6:2, v/v), ethyl acetate: propan-2-ol: water (10:7:6, v/v), propan-2-ol: 0.88 sp. g ammonia: water (12:6:2, v/v), acetone: 0.88 sp. g aqueous ammonia solution (25:1, v/v), chloroform: methanol: diethylamine (15:4:1, v/v) or methanol: water (9:1, v/v). Similarly the products and the isolated metabolite were identical using KC_{18} reversed phase TLC plates (Whatman, Maidstone, U.K.) developed in methanol:water (9:1, v/v). Samples of the metabolite and the reaction products were also identical when chromatographed by HPLC on a TSK gel LS410 column with acetonitrile: 0.02 M pH 6.3 phosphate buffer (1:4, v/v) as the mobile phase. The flow rate was 1 ml/min, detection was by U.V. absorbance at 296 nm and the analysis was carried out at ambient temperature. Isolation of the sulphate ester from a preparative reaction was carried out as follows. The sulphuric acid: tiaramide hydrochloride reaction mixture was titrated to pH 8.5 with 1.5 M sodium hydrogen carbonate. The product was adsorbed onto Amberlite XAD-2 resin, washed with distilled water and the crude product eluted with methanol. The eluate was evaporated to dryness under reduced pressure. The residue

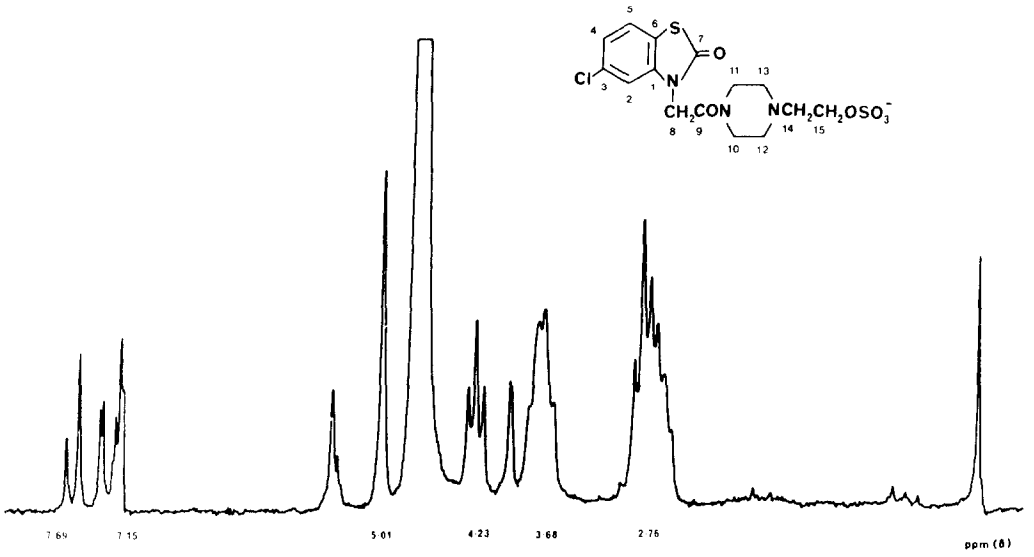

Fig. 2. Structure and (^{1}H) NMR spectrum of the isolated sulphate conjugate of tiaramide and the synthetic product. For assignment see text.

was then triturated with chloroform, dried and dissolved in a minimum volume of dimethylformamide (DMF). The product was precipitated from DMF with chloroform. The precipitate was triturated with chloroform and then hexane when it crystallised to yield a white solid. The overall yield of the reaction was 56%. The purified synthetic product was analysed by mass spectrometry under the same conditions as those used for the metabolite. The principal ions of the mass spectrum occurred at *m/e* 337, 324, 226, 198 and 170 in proportions similar to the spectrum of the isolated metabolite. Again no molecular ion was detected.

Proton NMR spectra of the metabolite and of the synthetic sulphate ester were obtained using a Bruker WP-80 spectrometer. The spectra were recorded in deuterium oxide containing sodium 2,2-dimethyl-2-silapentone-5-sulphonate as an internal reference standard. Both the metabolite and the synthetic sulphate ester gave identical spectra (Fig. 2).

Multiplet resonances at δ2.76 and 3.68 ppm in the spectra are assignable to the protons of the piperazine ring together with the methylene protons at position 14. The singlet resonance at δ5.01 ppm is due to the isolated methylene protons at the 8 position, whilst the resonances between δ7.15 and δ7.69 ppm arise from the aromatic protons. The triplet resonance at δ4.23 ppm in the spectrum of the metabolite, assignable to the protons at position 15, is shifted 0.67 ppm downfield with respect to the corresponding triplet in the spectrum of tiaramide. This downfield shift is consistent with deshielding of the protons by a strongly electronegative substituent. Such deshielding would be expected after the introduction of a sulphate ester grouping.

Field desorption mass spectrometry of the isolated metabolite and the synthetic product (Fig. 3) was conducted in a JEOL model JMS-D300 mass spectrometer equipped with JEOL FD/FI/EI combination ion source. The mass spectra of both were identical showing intense peaks at 436 and 458 which corresponded to the molecular ions of the sulphate conjugate (mol. wt 435) and its sodium salt. This result confirmed the identity of the metabolite isolated from female rat urine as the sulphate conjugate of tiaramide.

The spectroscopic, chromatographic and isotope experiments presented in this report confirm the identification of the major metabolite of tiaramide in female CR/CD and JCL/SD strain rats as the sulphate conjugate. The conjugate however was stable to arylsulphatase treatment which is to be expected for sulphate conjugates of aliphatic alcohols. Urinary and biliary excretion of this metabolite accounts for 55–65% of a 100 mg/kg dose of tiaramide. The conjugate is not detectable in the urine of males of these strains of rat although about 3–5% of the dose is excreted in the bile

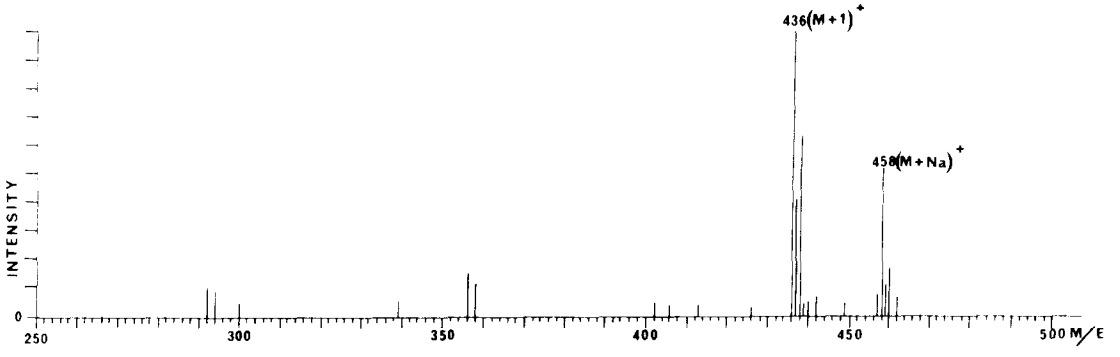

Fig. 3. Field desorption mass spectrum of the isolated sulphate conjugate of tiaramide and the synthetic product.

as this metabolite. It is known that aliphatic alcohols serve as substrates for sulphate conjugation [3]. The hydroxyethyl chain of tiaramide can be considered amenable, therefore, to sulphate conjugation. Lyon and Jakoby [4] have recently shown the identity of alcohol sulphotransferases with hydroxy steroid sulphotransferases. These enzyme activities have been shown to be sex-related and preferentially developed in female rats [5]. This to our knowledge is the first example of such a sex difference for sulphate conjugation being reported for a pharmaceutical agent.

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Adaptive modulations of brain membrane lipid fluidity in drug addiction and denervation supersensitivity

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The fluidity (reciprocal of microviscosity) of cell membrane lipids is implicated in many membranal functions, including transport [1], enyzmatic activity [2], receptor binding [3, 4], interaction of receptors with adenylate cyclase [5] and protein phosphorylation [6]. Under various conditions of stress which affect lipid fluidity (e.g. temperature changes), "homeoviscous adaptation" [7] is triggered so that lipid composition is altered to restore fluidity and proper membrane functions. Addictives, such as alcohol and barbiturates, have been found to have a fluidizing effect on membrane lipids [8]. In alcohol-addicted animals, tolerance is accompanied by restoration of membrane lipid fluidity by increased cholesterol level [9, 10]. Similarly, morphine has been shown, both *in vivo* and *in vitro*, to increase the lipid fluidity of synaptic membranes in a dose-dependent manner which can be blocked by naloxone [11], and to alter lipid metabolism [12].

In this study, we have investigated whether tolerance to, and dependence on, morphine may also involve a process of homeoviscous adaptation in an animal model. In parallel, we have studied the effect of a related phenomenon, denervation supersensitivity, on changes in the lipid microviscosity of brain membranes.

Morphine (HCl) was obtained from Assia (Petach Tikvah, Israel), naloxone from Endo Laboratories (Garden City, NY), 1,6-diphenyl-1,3,5-hexatriene (DPH) from Koch-Light Laboratories (Colnbrook, England), and Tris free base ("trizma") from the Sigma Chemical Co. (St. Louis, MO). Animals (Sabra rats, 2- to 3-months-old) were injected intraperitoneally with morphine (HCl) or with naloxone in doses described below. Alternatively, the animals were implanted subcutaneously with 75 mg morphine (free base) pellets according to Way *et al.* [13]. The times from treatment to decapitation are given below.

Fornix lesions were produced unilaterally, and the location of the lesion was verified histologically. The animals were decapitated 30 days after the lesions were made, and the brains were removed and dissected into regions. Nigrostriatal lesions were produced unilaterally according to Ungerstedt [14], and only animals that showed intensive circling towards the intact side when challenged with 0.5 mg/kg apomorphine were tested. The animals were decapitated 30 days after the lesions were made. The brains were removed and dissected into regions. The tissues from the various brain regions were gently ground with a Teflon pestle and vortexed. The heavy particles were allowed to settle, and the upper liquid was transferred to another set of tubes and centrifuged at 1500 rpm for 10 min. Microscopic examination showed that the pellet contained a mixture of dissociated cells and cell clusters with some cell debris. This preparation was used in most membrane fluidity measurements. Although the results only partially represent plasma membrane microviscosity, the measurements proved to be much more reproducible with this preparation than with partially purified membrane preparations (see below).

Membrane lipid microviscosity ($\bar{\eta}$—the reciprocal of fluidity) was determined by fluorescence depolarization using the lipid probe, DPH (for a review, see Ref. 15). A stock solution of 2 mM DPH in tetrahydrofuran was diluted 1:1000 in vigorously stirred phosphate-buffered saline (pH 7.2). A sample (0.5 to 1 mg) of dissociated cells was sus-