Xenobiotica
the fate of foreign compounds in biological systems

ISSN: 0049-8254 (Print) 1366-5928 (Online) Journal homepage: http://www.tandfonline.com/loi/ixen20

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To cite this article: J. C. Ritchie, S. C. Mitchell, R. L. Smith & A. Q. Zhang (1996) Use of site-specified tritium labelling to confirm the formation of 17-oxosparteine as a minor urinary metabolite of sparteine in man, Xenobiotica, 26:9, 977-982

To link to this article: http://dx.doi.org/10.3109/00498259609052499

Published online: 27 Aug 2009.

XENOBIOTICA, 1996, VOL. 26, NO. 9, 977–982

Use of site-specified tritium labelling to confirm the formation of 17-oxosparteine as a minor urinary metabolite of sparteine in man

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Received 1 December 1996

1. The synthesis of [*17,17*-$^3H_2$]-sparteine and its oral administration has enabled the specific identification of 17-oxosparteine as a minor urinary metabolite ($\approx$ 1 % dose) in two healthy male volunteers.

Introduction

Sparteine is a naturally occurring quinolizidine alkaloid widely distributed amongst leguminous plants of the genera *Baptisia*, *Cytisus* and *Lupinus*, and readily extracted by steam-distillation from scoparium, the ground dried tops of the common broom (*Cytisus* [*Sarothamnus*] *scoparius*). The sulphate salt has been employed as a cardiac stimulant (Raschack 1974) and as an oxytocic for the induction of labour and the treatment of uterine inertia (Brazeau 1965), but its clinical usage has been curtailed by unpredictable adverse reactions typical of overdose (Newton *et al.* 1966). The reasons for such problems became clearer when it was shown that sparteine was polymorphically metabolized by cytochrome P4502D6 (Price Evans 1993) with some individuals excreting virtually all of the administered dose unchanged in the urine (Eichelbaum *et al.* 1975, 1986). Metabolism, when it occurred, appeared limited with 2-dehydrosparteine and 5-dehydrosparteine being identified as the major urinary products, presumably arizing from reactive intermediates via rearrangement and dehydration. However, it has been suggested that other metabolites (or degradation products of the dehydrosparteines) are also excreted in the urine and various dimer configurations have been proposed (Eichelbaum *et al.* 1986). During studies on the polymorphic nature of sparteine metabolism we had occasionally noted an alkaloid-positive area, which tailed just behind sparteine on thin-layer plates (solvent A below) and which co-chromatographed with authentic 17-oxosparteine. We now present evidence, involving the specific use of [*17,17*-$^3H_2$]-sparteine to show that 17-oxosparteine is a minor urinary metabolite of sparteine in man.

Materials and methods

Chemicals

(−)-Sparteine (figure 1) (lupinidine; dodecahydro-7,14-methano-2*H*,6*H*-dipyrido[1,2-*a*:1′,2′-*e*][1,5]diazocine) sulphate pentahydrate ($C_{15}H_{28}N_2O_4S$) was obtained from Sigma Chemical Co. (Poole, UK). Its purity ($\geqslant$ 99 %) was confirmed by capillary gc/ms and on heating it looses water at 100°C, turning brown, m.p. 136°C dec.(uncorr.)

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0049–8254/96 $12·00

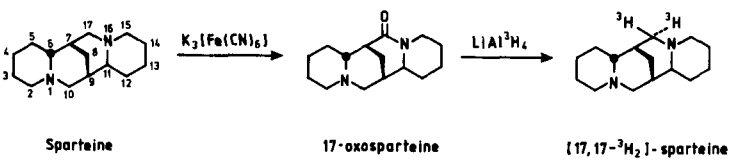

Figure 1. Chemical structures of sparteine, 17-oxosparteine and the synthesis route for [*17,17*-$^3H_2$]-sparteine.

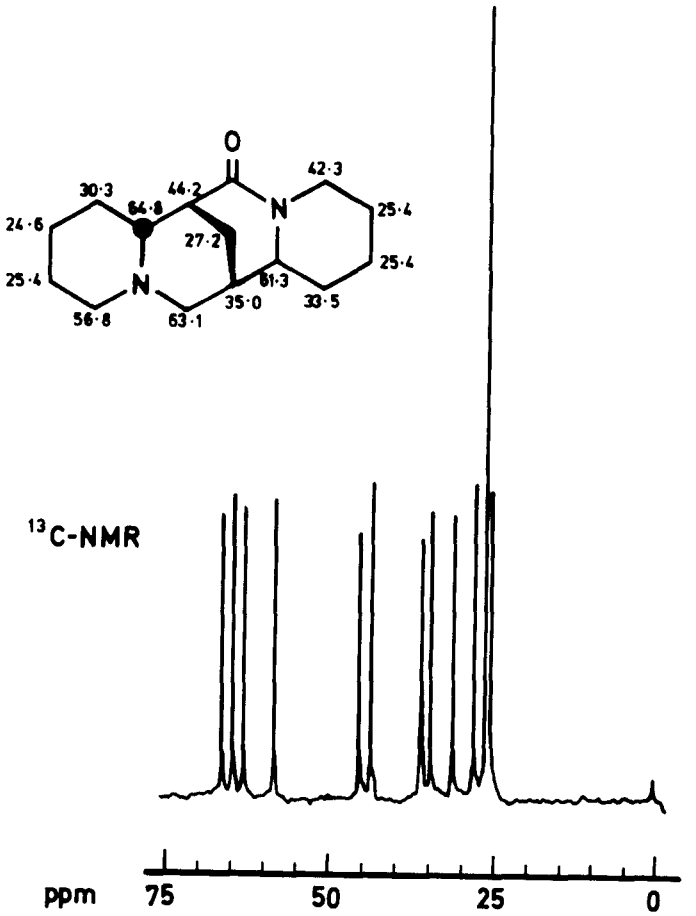

Figure 2. [^{13}C]-nmr spectrum and signal assignments (ppm) for 17-oxosparteine.

17-Oxosparteine (figure 1) was synthesized in the laboratory. A cold aqueous solution (20 ml) of (−)-sparteine sulphate (20 g) was added slowly to a mixture of potassium ferricyanide (80 g) and sodium hydroxide (12·8 g) previously dissolved in water (300 ml) and held on ice at 0°C. Following further stirring at room temperature for 30 min, diethyl ether extraction (3 × 300 ml) was undertaken. The combined diethyl ether extracts were then dried over anhydrous calcium chloride before being evaporated under reduced pressure (Clemo *et al.* 1936). The residue obtained was repeatedly recrystallized from *n*-hexane to produce white crystals, m.p. 87°C (uncorr.) (lit. values: 86–87°C, Clemo and Raper 1929; 83–84°C, Ahrens 1891), with a chemical yield (as free base) of 56·1%. Elemental analysis; expected for $C_{15}H_{24}N_2O$ requires, C 72·6%, H 9·7%, N 11·3%; found C 72·9%, H 9·8%, N 11·3%. Infrared spectra gave characteristic bands at 2800–2700 cm^{-1} as previously described for 17-oxosparteine (Bohlmann 1958). Fourier transform [^{13}C]-nmr spectroscopy gave signals which are illustrated together with their assignments (figure 2), these positions being in agreement with literature values (Bohlmann and Zeisberg 1975).

The synthesis of [*17,17*-$^3H_2$]-sparteine was undertaken as follows. In dry apparatus, 17-oxosparteine (0·4 mmol; 100 mg) was added slowly to lithium aluminium [^{3}H]-hydride (1·2 mmol; 5 mCi) (New

England Nuclear, Boston, MA, USA) in tetrahydrofuran (1·2 ml) and stirred on ice for 60 min. A calcium chloride tube was employed to prevent the ingress of moisture. After this period, crushed ice (*c.* 20 ml) was added, mixed (*c.* pH 12), and the reaction products extracted with cold diethyl ether (4 × 50 ml). Following filtration, the combined diethyl ether layers were evaporated to dryness and the residue dissolved in a minimum volume of ethanol (*c.* 2 ml) (Binnig 1974, Clemo *et al.* 1949, Bohlmann *et al.* 1957, Bohlmann 1958). The radioactive sparteine was purified by preparative thin-layer chromatography in solvent B (see below), with the appropriate area (located by co-chromatography and spraying adjacent strips with iodoplatinate reagent: sparteine R_f = 0·08–0·12, 17-oxosparteine R_f = 0·84–0·88) being eluted with acidified methanol (1 % v/v 2 M H_2SO_4; 4 × 25 ml), filtered to remove silica and then evaporated to dryness. A small amount of this product was removed for analysis for capillary gc-ms before the remainder was co-crystallized from ethanol with non-radioactive sparteine (400 mg). This final product was also analysed by gc-ms.

Gc-ms analyses showed co-chromatography with authentic (−)sparteine, a chemical purity > 99·9 % and the absence of 17-oxosparteine to 0·01 % by weight. The mass spectra obtained agreed with literature values (Wink *et al.* 1981, Pospísil *et al.* 1992) and that obtained for authentic sparteine. Chemical and radiochemical purity by tlc (see below) was > 99·8 % with a specific activity of 18·9 uCi/mmol (1 uCi/22·4 mg). The radiochemical yield of this reaction was very low (*c.* 1 %) but nevertheless provided sufficient [*17,17*-$^3H_2$]-sparteine for two human dosings.

Volunteer studies

Two healthy male subjects (29 and 31 years), who had previously been suspected of producing this metabolite, volunteered for this study after given written informed consent, ethical approval being obtained from the local Ethics Committee. The subjects were non-smokers and neither alcohol nor medication were permitted for 1 week before or during the study. After collecting a control 0–24 h urine sample, they were given a single oral dose of radiolabelled sparteine (170 mg equivalent to 94·3 mg free base; 7·59 μCi) dissolved in water (20 ml) during the early morning about 1 hour after a light breakfast. Urine and faeces were collected at known intervals over the following 3 days.

Chromatography

Tlc was undertaken on silica gel G and 60 F_{254} plates (0·2 mm thick; 20 × 20 cm glass and aluminium backed respectively) and developed in solvent A [ethyl acetate/methanol/aq.NH_4OH (sp. gr. 0·88) (10/1/1 by vol.)]. Preparative tlc was undertaken on silica gel G plates (2 mm thick) in solvent B [chloroform/methanol/aq.NH_4OH (sp. gr 0·88) (90/9/1 by vol.) (Cho and Martin 1971)].

Gc-ms was undertaken on a Hewlett Packard 5890 II series gas chromatograph fitted with a 5971 series mass selective detector (Hewlett Packard, Stockport, UK). The fused-silica capillary column (30 m × 0·25 mm id) was coated (film thickness 0·25 μm) with cross-linked phenyl-methyl silicone (5 %) with a helium gas flow-rate of 1 ml/min. The column oven was initially held at 70°C for 2 min, then raised at 10°C/min until a temperature of 275°C was reached, which was maintained for a further 3 min. The injector port was held at 250°C. The gc-ms interface temperature, the ionization energy and the ion source temperature of the electron impact mass spectrometer was 280°C, 70 eV and 175°C respectively. During instances when the mass selective detector was not activated, a flame-ionization detector held at 280°C was employed.

Determination of radioactivity

Aliquots of urine (1·0 ml), and areas of thin-layer chromatograms (see later), were added directly to vials containing scintillation fluid (20 ml, 'Ecoscint'; National Diagnostics, Atlanta, GA, USA) and counted by liquid scintillation spectrometry using a Packard Tri-Carb 4640 scintillation counter (Canberra-Packard Instrument Co., Meriden, CT, USA) with an external standard being used for quench correction. Known volumes of urine were lyophilized and the dried residue reconstituted to the original volume with water. Determination of the loss in radioactivity per unit urine volume gave a measure of the degree of tritium exchange occurring from sparteine to water. Faeces was homogenized with water, lyophilized, ground to a powder, weighed, and quintuplicate samples (about 75 mg) combusted in O_2 (Harvey Biological Material Oxidiser, ICN Tracer Labs, High Wycombe, UK), the ^3H_2O so produced being trapped in an alkaline diphenylethylamine-containing scintillation cocktail (Patterson and Greene 1965, Peterson *et al.* 1969).

Metabolite determination

The 0–12-h urine was chosen for metabolite study. Concentrated diethyl ether extracts (× 5 vol.) of alkaline (KOH; pH 12) urine (5 ml) were applied either as small roundish areas or streaks (50–200 μl) to thin-layer plates and developed in solvent A. Some plates were sprayed with the iodoplatinate reagent (Elliott 1959, Barnsley *et al.* 1964, Cho and Martin 1971) whilst others were divided into 0·3-cm strips, the silica removed, placed into scintillation vials and counted for radioactivity as described above.

Metabolite areas under specific investigation were eluted with methanol, centrifuged and concentrated under a stream of nitrogen before examination by mass spectrometry. The use of known amounts of 17-oxosparteine added to control urine and taken through the above extraction, chromatography and subsequent elution procedures permitted the construction of calibration curves and enabled quantitation.

Physical chemistry

Infrared spectrometry was carried out on potassium bromide discs employing a Perkin Elmer 157G grating infrared spectrophotometer (Perkin Elmer, Beaconsfield, UK).

In addition to the Hewlett Packard 5971 series mass selective detector attached to the capillary gas chromatograph, electron impact mass spectrometry was also carried out on a Kratos MS80 instrument (Kratos, Manchester, UK) with Kratos D555 (data generator) computerized display and printout facilities. The samples were inserted directly into the ionization chamber at 70 eV with a source temperature of 200°C.

Pulsed Fourier transform [^{13}C]-nmr spectroscopy was carried out in $CDCl_3$ (deuterium locked) using a JEOL (FX60/804) machine (JEOL Europe BV, Brussels, Belgium) with chemical shifts (ppm) being measured relative to the tetramethylsilane internal reference standard following proton noise decoupling.

Results and discussion

Within 12 h of [*17,17*-$^3H_2$]-sparteine administration a mean of 31·4% (29·2; 33·5) of the radiolabel was excreted in the urine and these collective samples were used for further examination. Fractional urine collection showed that within the first 3 h post-dose, 18·9% (16·5; 21·3) of the administered radioactivity was eliminated, with 26·0% (23·8; 28·2) being voided after 6 h and 29·0% (26·5; 31·5) after 9 h. Between the urine and faeces a complete recovery (97·9% {98·4; 97·4}) was achieved after 72 h.

Tlc of diethyl ether extracts of alkaline urine (0–12 h) showed several iodoplatinate-positive areas (grey/black areas on a white ground), which were coincident with radioactivity. Co-chromatography tentatively identified two of these radioactive areas as sparteine (R_f = 0·71, solvent A) and 5-dehydrosparteine (R_f = 0·11). A third area was presumably 2-dehydrosparteine (R_f = 0·33) although no authentic standard was available. The two dehydro metabolites gave a red colouration with the nitroprusside reagent, previously noted for Δ^5 and Δ^{11} dehydro compounds (Elliott 1959, Cho and Martin 1971), whereas sparteine did not react.

However, one iodoplatinate-positive area (R_f = 0·66), not present in control urine, and running just below sparteine (R_f = 0·71) was not coincident with an area of radioactivity, indicating the removal of tritium atoms. Co-chromatography suggested that this metabolite may have been 17-oxosparteine. Elution of this tlc area with methanol and subsequent examination by gc-ms showed the presence of one major peak (R_t = 19·7 min), which co-chromatographed with authentic 17-oxosparteine and gave a mass spectrum which was diagnostic (Wink *et al.* 1981; Pospísil *et al.* 1992). The fragmentation pattern of this compound arises mainly by cleavage of the central eight-membered ring structure and it is known that the oxosparteines show fundamental and distinctive differences in their fragmentation patterns (McLafferty and Stauffer 1989). A second smaller peak (R_t = 16·1 min), identified as sparteine, was also present and presumably arose from the incomplete separation of the two compounds (17-oxosparteine and sparteine) by tlc. Both of these peaks were distinct from that generated on gc-ms by authentic 2-oxosparteine (R_t = 20·6 min) (figure 3).

In both subjects 17-oxosparteine accounted for about 1% of the administered dose (0·9; 1·2%). These values were several orders of magnitude greater than the impurity levels of this compound in the initial [*17,17*-$^3H_2$]-sparteine ingested. Such low levels are in agreement with a single previous report where up to 1·7% of the

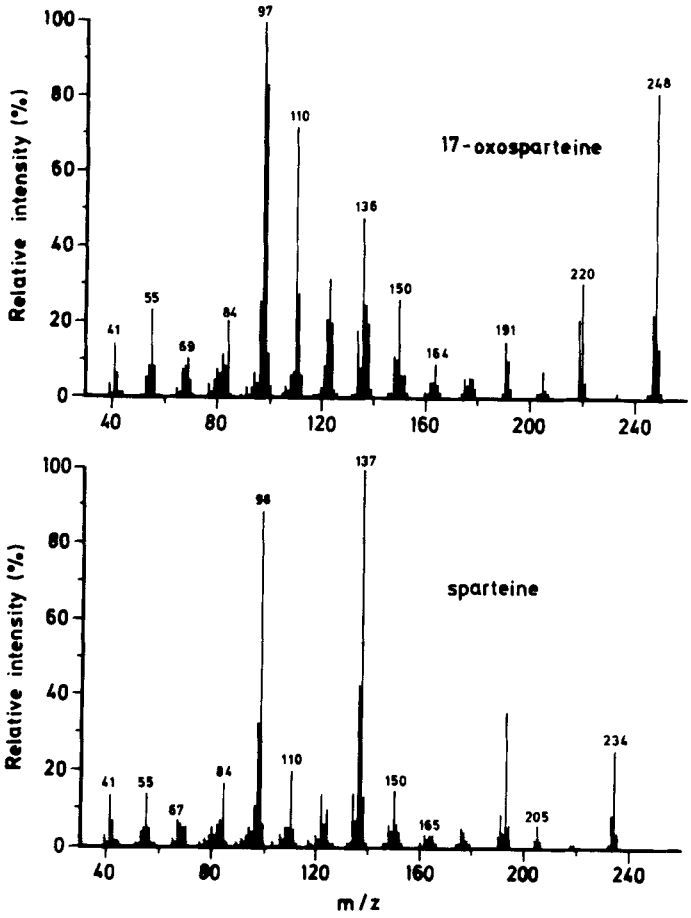

Figure 3. Electron-impact mass spectral fragmentation patterns obtained for authentic 17-oxosparteine and sparteine via capillary gc-ms.

total recovery (dose 100 mg sparteine sulphate) was identified as 17-oxosparteine. Similar amounts of 2-oxosparteine were also detected and by examining metabolite ratios these authors suggested that the dehydrosparteines may be the first metabolic step in the formation of the oxysparteines or that there exists some common intermediate (Pospísil *et al.* 1992). From our accumulated experience, although the above two volunteers produced 17-oxosparteine, many individuals similarly examined have not shown signs of this metabolite.

Acknowledgements

We thank Dr M. Eichelbaum, Dr Margarete Fischer-Bosch-Institut für Klinische Pharmakologie, Stuttgart, for his generous gift of 5-dehydrosparteine, and the Department of Chemistry, University of Birmingham, UK, for undertaking the nmr analyses.

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