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Note

Simultaneous determination of sparteine and its 2-dehydro and 5-dehydro metabolites in urine by high-performance liquid chromatography with electrochemical detection

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Sparteine is a natural alkaloid with antiarrhythmic and oxytocic properties, but is rarely used because of the severe effects multiple dosage generates in some individuals. Genetic deficiencies in the oxidation of sparteine, which appear to be the reason for the severe effects, were initially reported on by Eichelbaum et al. [1]. Spiteller and Spiteller [2] reported that sparteine is metabolized by cytochrome P-450 through N_1-oxidation to its unstable N_1-oxide which rearranges, with dehydration, to give 2-dehydro-and 5-dehydrosparteine with 2-dehydrosparteine as the major product (Fig. 1). However, it has recently been reported that they may be formed through carbinolamines [3] or, perhaps, through α-hydroxysparteine [4]. Bertilsson et al. [5] and Inaba et al. [6] have shown that the biotransformation defect which results in poor metabolism of sparteine is generated from the same cytochrome P-450 defect which results in poor metabolism of debrisoquine [7].

To investigate the rate of formation of these metabolites and to determine the overall metabolic rate for sparteine exhibited by individuals in a population, reliable and sensitive assay methods for sparteine, 2-dehydrosparteine and 5-dehydrosparteine are necessary.

Initially, Eichelbaum et al. [8] used packed-column gas chromatography

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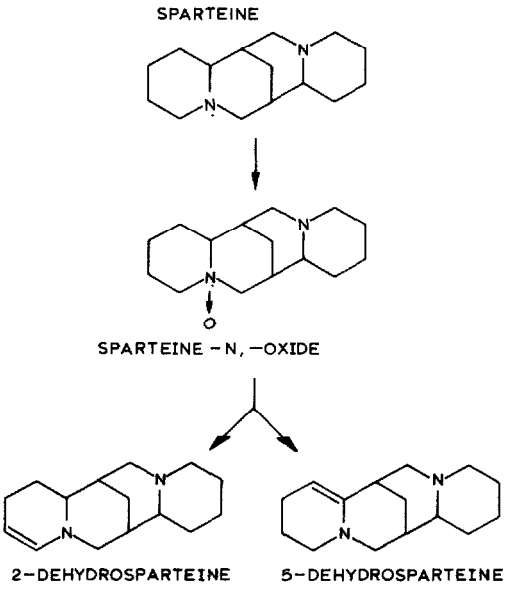

Fig. 1. Structures of sparteine and its major metabolites.

(GC) with flame ionization detection (FID) for the assay of sparteine and its metabolites in urine after basic organic extraction of the analytes. Later it was found that, because of adsorption on the column support, this method yielded only 50% of the metabolites relative to sparteine itself, at low levels, and sparteine had originally been used as the reference material for both sparteine and its metabolites in the assays [9]. By concentrating poor metabolizer samples ten-fold and using metabolite standards rather than just sparteine standards, lower metabolite concentrations, previously undetected, were detected in most cases, giving lower metabolic ratios (MRs) for poor metabolizers than previously found [9,10]. This sensitivity has now been further increased by using capillary GC with nitrogen–phosphorus detection (NPD), but still requires organic solvent extraction [11]. Vinks et al. [12] modified the extraction system in the method of Eichelbaum et al. [8] and used nitrogen-selective detection rather than the less sensitive FID, to assay sparteine and its metabolites in urine using standards of each analyte. Otton et al. [13] used the original inefficient extraction system [8] and the GC method used by Vinks et al. [12] to assay sparteine and its metabolites in liver homogenates. Sparteine isomers in an extract of above-ground portions of *Lupinus argenteus* have been separated and identified at high concentrations by Keller et al. [14] using thin-layer chromatography (TLC) and gas chromatography–mass spectrometry (GC–MS). Inaba et al. [6] used sodium borohydride to reduce the sparteine metabolites back to sparteine. However, the method converts both metabo-

lites, so only total metabolites can be estimated from the difference between sparteine levels in borohydride-treated and untreated urine samples, and each sample has to be assayed twice.

Thus a method which eliminates the organic extraction procedure, assays sparteine and each metabolite simultaneously and is sufficiently sensitive to assay the metabolites in poor metabolizer urine at concentrations three orders lower than occurs in extensive metabolizers, would greatly assist in the use of this drug for phenotyping purposes.

The method reported herein utilises high-performance liquid chromatography (HPLC) in the reversed-phase mode with electrochemical detection to fulfill these requirements.

EXPERIMENTAL

Reagents and standards

All solvents were spectroscopic grade and all water used was purified by the Milli-Q system (Millipore, Milford, MA, U.S.A.). All other reagents were analytical-reagent grade. The sample and elution buffer was 0.01 *M* sodium dihydrogenphosphate with 700 μl/l of 86% (w/v) orthophosphoric acid (pH 2.5). 2-Dehydrosparteine, 5-dehydrosparteine and 17-ethylsparteine were kindly donated by Prof. Dr. M. Eichelbaum (Dr. Margarete Fischer-Bosch-Institut, Stuttgart, F.R.G.). The internal standard solution was 40 μg/ml 17-ethylsparteine in sample buffer.

Sample collection

Three drug-free male Caucasian members of the departmental staff were the subjects used in the initial investigation of the analytical methodology. After emptying the bladder for drug-free sample collection, sparteine (100 mg sparteine sulphate, Depasan) was administered orally to each fasting subject and the total urine collected for 0–8 h post-dose. Aliquots (20 ml) of the measured 8-h collection were frozen in liquid nitrogen and stored at −18°C until assay.

Sample preparation

A 100-μl volume of urine was added to 50 μl sample buffer plus 50 μl internal standard solution (40 μg/ml 17-ethylsparteine in sample buffer), vortex-mixed for 5 s and injected into the chromatograph through a 25-μl loop.

Preparation of standards

A 50-μl volume of standard in sample buffer (range 0.1–50 μg/ml) was added to 100 μl drug-free urine with 50 μl internal standard solution, mixed on the vortex-mixer and injected as for the sample urines.

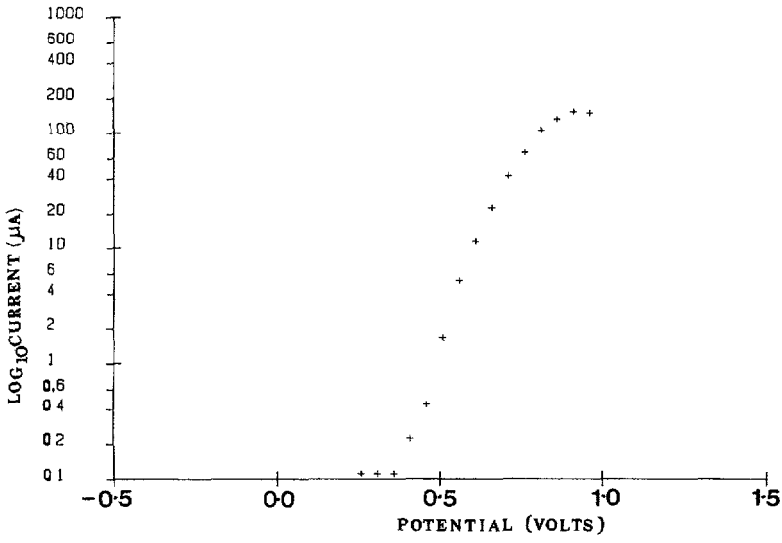

Fig. 2. Hydrodynamic voltammogram of sparteine in mobile phase (pH 2.5) after correction for mobile phase effect.

Chromatography

HPLC separation was performed on a Spectra Physics 8100 liquid chromatograph fitted with a Valco loop valve and a 25-μl loop. Separation was achieved isocratically on a 250 mm$\times$4.6 mm I.D. Spherisorb S5 CN column (5 μm particle size) with acetonitrile–methanol–pH 2.5 phosphate buffer (25:32:43, v/v) as the mobile phase at a flow rate of 2 ml/min. The column temperature was 28°C. All solvents were degassed ultrasonically and then sparged and maintained under helium.

Detection

Coulometric detection was effected with a high-sensitivity dual-electrode cell (Model 5011) ESA Coulochem detector (Model 5100A) in the oxidative screen mode with detector I at a potential of +0.3 V and detector II at +0.9 V, as suggested by the dynamic voltammogram of sparteine in mobile phase (Fig. 2). The detector responses were recorded simultaneously on a Perkin Elmer 56 strip-chart recorder and a Spectra Physics SP 4200 integrator.

RESULTS

Using the above technique, good separation and detectability of the analytes in urine were achieved, free from endogenous component interference, as can be seen in the chromatograms (Fig. 3). No interfering components were found in the drug-free urine. A study [15] in 97 Southern African Barakwena sub-

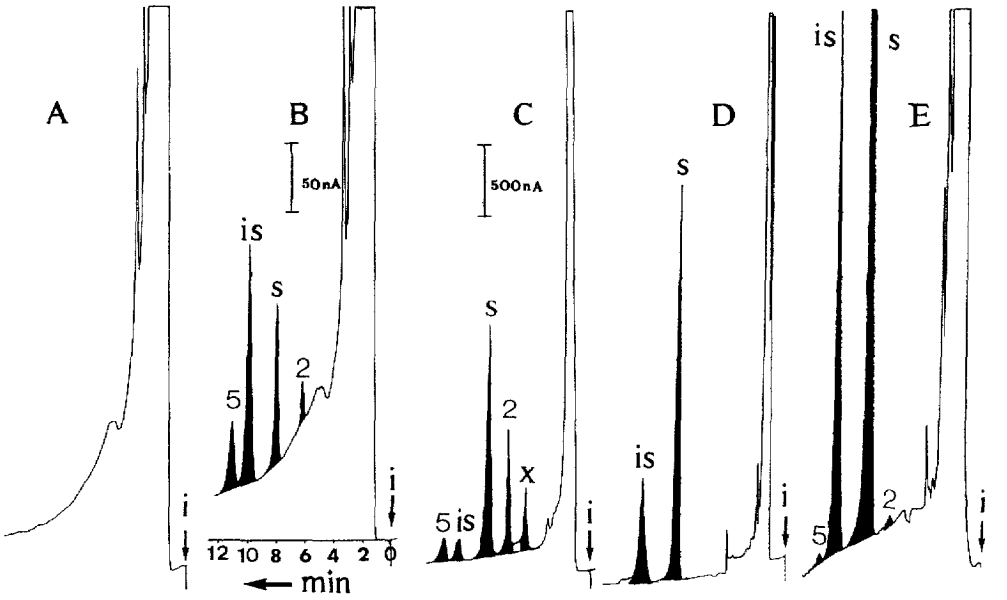

Fig. 3. Chromatograms of urine and standard samples. (A) Drug-free urine. (B) Standard sample containing 2 μg/ml 2-dehydro- and 5-dehydrosparteine, 1 μg/ml sparteine and 40 μg/ml 17-ethylsparteine (internal standard). (C) Extensive metabolizer urine collected for 8 h after oral dosing with 100 mg sparteine sulphate. (D) Poor metabolizer urine after same dosing and collection. (E) Same poor metabolizer urine, at higher sensitivity. Peaks: i = inject; 2 = 2-dehydrosparteine; 5 = 5-dehydrosparteine; s = sparteine, is = internal standard; x = unidentified peak which occurs in dosed samples from some extensive metabolizers.

jects also showed no co-eluting components in the drug-free urine and showed an incidence of 4.1% poor metabolizers with a bimodal phenotype distribution in this negroid population. This value lies between the incidence of 5–8% in Caucasians [16], 2.4% in Japanese [17] and no poor metabolizers in Ghanaians [18]. Standard response curves were linear over the ranges 1–50 μg/ml (40 μg/ml 17-ethylsparteine as internal standard) and 0.1–5 μg/ml (4 μg/ml 17-ethylsparteine as internal standard). Above 50 μg/ml the detector response plateaued due to saturation of the oxidising surface. Thus, samples showing concentrations above 50 μg/ml were diluted to be within the linear range and reassayed. The inter-assay standard deviation over six samples was better than 3% for sparteine at 25 μg/ml, but deteriorated to worse than 10% at 200 μg/ml. For 2-dehydrosparteine the inter-assay standard deviation was better than 3% and better than 1% for 5-dehydrosparteine at 50 μg/ml.

The limit of determination was 4 ng/ml for sparteine, 15 ng/ml for 2-dehydrosparteine and 20 ng/ml for 5-dehydrosparteine using a 25-μl loop. The limit of detection was 2 ng/ml for sparteine at a signal-to-noise ratio of 3. The detectability increased almost four-fold if a 100-μl loop was used.

DISCUSSION

Initial investigation showed that sparteine absorbs poorly in the ultraviolet and visible region and does not fluoresce. Since assay at the ng/ml level is required to estimate metabolic rates in poor metabolizers, ultraviolet detection is obviously not applicable in this case,

The presence of two tertiary nitrogens in sparteine (Fig. 1) suggested the use of electrochemical detection and this was investigated. Voltammograms of sparteine (Fig. 2) revealed that a high oxidation potential of +0.9 V would be needed to yield a quantitative oxidation of sparteine. Coulometric detection was used, since the large surface area of the coulometric electrode is supposed to be less susceptible to the fouling which occurs at high voltages and gives a much larger response than the smaller electrode surface of an amperometric detector. Thus, coulometric detection is particularly indicated at the high oxidation voltage required. Consistency of the buffer salt content was found to be essential for highly reproducible results between buffer lots.

It was found that within the column pH range of 2–8, sparteine did not elute quickly enough from a 250 mm×4.6 mm I.D. Spherisorb S5 ODS2 (5 μm particle size) column to give sufficient sensitivity, even when using a 70% (v/v) organic modifier mobile phase. Elution was found to be satisfactory on a less lipophilic 250 mm×4.6 mm I.D. Spherisorb S5 CN (nitrile) (5 μm particle size) column, and this was used for the final method development. Extensive tailing occurred above pH 4. Best peak shape and detection were at pH 2.5, and the peak height for sparteine was four times that of the already partially oxidised metabolites at the same molar concentration.

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