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Note

Gas chromatographic determination of sparteine and 2- and 5-dehydrosparteine in plasma and urine

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Sparteine (SP) in man is a widely applied substrate probe for the characterization of individual subjects as poor metabolizers (PM) or extensive metabolizers (EM) of the drug. SP is metabolized by the cytochrome P-450 system to two major metabolites, 2- and 5-dehydrosparteine (DHS), which are excreted into the urine. The metabolic oxidation of SP exhibits polymorphism, which was first described by Eichelbaum and co-workers [1,2]. Subsequent studies revealed that this polymorphism cosegregates with debrisoquine and affects 5–10% of the Caucasian population [3,4]. Family studies clearly demonstrated the genetic basis for this phenomenon and that the inheritance is autosomal recessive [4,5].

Analysis of SP and DHS is almost exclusively carried out on urine samples, because the ratio of SP to DHS, as determined in 0–8 or 0–12 h urine samples, allows the assessment of the oxidation phenotype [6]. However, this ratio gives neither quantitative information on clearance of parent drug nor on the rate of formation of the metabolites, parameters which are directly related to enzyme activities [7]. Estimation of oral clearance based on the excretion rate of SP in urine only [8] is inappropriate, because the drug is subject to considerable first-pass oxidation.

For the determination of SP and DHS in urine, several gas chromatographic methods have been described [9–12], based on flame-ionization detection. Assays have been published for SP and DHS in plasma [2,12], but limited analytical methodology is available for the determination of the relatively low concentrations of SP and 2- and 5-DHS in plasma, which is required when SP is administered in low doses.

In this paper a selective and sensitive assay is described for SP and 2- and 5-

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DHS in plasma and urine and some preliminary results obtained with poor and extensive metabolizers of SP are reported.

EXPERIMENTAL

Chemicals

SP (7,14-methano-2*H*-dipyrido[1,2-*a*:1′,2′-*e*][1,5]diazocine) was obtained as SP sulphate from Fluka (Buchs, Switzerland). 5-DHS (Δ^5-DHS) was a generous gift from Dr. M. Eichelbaum (Dr. Margarete Fischer-Bosch Institute, Stuttgart, F.R.G.). Tolazoline was obtained from OPG (Utrecht, The Netherlands) and benzphetamine from Upjohn (Kalamazoo, MI, U.S.A.). Sodium borohydride, methanol (analytical-reagent grade) and all other chemicals were obtained from J.T. Baker (Deventer, The Netherlands).

Apparatus

The gas chromatographic system consisted of a Hewlett-Packard Model 5710A gas chromatograph, equipped with a nitrogen–phosphorus selective detector (rubidium crystal). The injection port consisted of an all-glass closed system with a falling needle device for solid injection. The injection port and detector temperatures were 300°C and the column temperature was 180°C. Helium was applied as the carrier gas at a flow-rate of 3–6 ml/min; flow-rates for detection were hydrogen 6 ml/min and air 30 ml/min.

A glass capillary column (15 m × 0.30 mm I.D.) was used and the dynamic coating procedure adapted from Van der Graaff et al. [12] was applied. A support layer of Tullanox (silanized fumed silica; Cabot, Boston, MA, U.S.A.) of 10 μm particle size was deposited, followed by flushing of the column with a 1% solution of potassium hydroxide in methanol. Carbowax 20M was used as the stationary phase; the column was flushed twice with a 2% solution of Carbowax in dichloromethane. Nitrogen gas was flushed through the column to dry it.

All data were processed with a Shimadzu (Kyoto, Japan) CR3-A integrator. A Buchler (Fort Lee, NJ, U.S.A.) vortex evaporator was used to mix the samples and evaporate them to dryness.

Assay of drug and metabolites in plasma

For the determination of SP and 2- and 5-DHS, 50 μl of 4 *M* sodium hydroxide solution, 50 μl of tolazoline as internal standard (68.4 ng per 100 μl) and 5 ml of DCM–*n*-pentane (1:1, v/v) for extraction were added to 0.50 ml of plasma. After mixing for 30 min the sample was centrifuged at 2000 *g* and the organic layer was collected and evaporated nearly to dryness. The residue was dissolved in 100 μl of methanol and 2 μl of the solution were deposited on the glass needle of the injection port to evaporate during 1 min. Subsequently the dry sample was injected. Peak areas were used for quantification of the compounds.

Assay of drug and metabolites in urine

The assay of SP and 2- and 5-DHS was carried out according to the borohydride reduction procedure [12,13] with some modifications. To 0.50 ml of urine, 50 μl

of tolazoline as internal standard (5 μg per 100 μl), 50 μl of 4 *M* sodium hydroxide solution and 5 ml of DCM–*n*-pentane (1:1, v/v) were added. After mixing for 30 s on a Whirlmixer and centrifugation at 2000 *g* for 5 min, the organic layer was taken and evaporated nearly to dryness. The residue was dissolved in 200 μl methanol and 1 μl of the solution was injected. DHS metabolites were determined as SP after reduction of a urine sample with sodium borohydride. To 0.50 ml of urine 1 mg of sodium borohydride was added and this mixture was left for 30 min at room temperature. DHS was measured as the difference between the SP concentration before and after reduction. Peak heights were used for quantification of the compounds.

Preparation of calibration graphs

Plasma. SP solution was added to different test-tubes (10, 20, 40, 70, 100, 150 and 200 μl of a 25 ng per 100 μl solution) and evaporated nearly to dryness, followed by addition of 0.50 ml of plasma. Subsequently, the samples were carried through the analytical procedure. 5-DHS was also applied for the detection of 2-DHS, because no standard was available. Identical extraction behaviours and detector sensitivities for 2- and 5-DHS were assumed. For calibration graphs 5-DHS was added to test-tubes (5, 10, 20, 30, 40, 80 and 150 ng) analogously to SP.

Urine. SP solution was added to different test-tubes (5, 10, 20, 40, 60 and 80 μl of a 2.77 μg/ml solution) and, after evaporation nearly to dryness, 0.50 ml of urine was added.

An additional series was prepared for the borohydride reduction in order to determine DHS excretion.

Human experiments

In this preliminary study 50 mg of SP (as sulphate) were administered orally, after an overnight fast, to a previously phenotyped EM and to a PM of SP, on the basis of urinary excretion ratio. SP was administered in a "cocktail approach" [14] together with nifedipine (5 mg) and mephenytoin (100 mg). Prior to the study the volunteers had been medically examined and they had given their written informed consent. Blood samples were taken ($n=14$) from a fore-arm vein at regular intervals from 0 to 32 h after drug intake. Urine was collected in three portions up to 32 h. All samples were kept at −20 °C until analysis.

RESULTS AND DISCUSSION

Assay in plasma

Typical chromatograms obtained after extraction of blank plasma and a sample taken from a subject during the study, containing 175 ng/ml SP, 25.8 ng of 5-DHS and 272 ng of 2-DHS, are shown in Fig. 1. No interfering compounds seemed to have been co-extracted. The retention times were 2.9 min for SP, 4.1 min for 5-DHS, 4.6 min for 2-DHS and 6.5 min for tolazoline. The calibration graphs for SP were linear in the concentration range 1–50 ng/ml. The detection limit of SP in plasma was approximately 200 pg/ml and the coefficients of variation were 3% for SP, 2% for 2-DHS and 4% for 5-DHS as determined at the mid-points of the

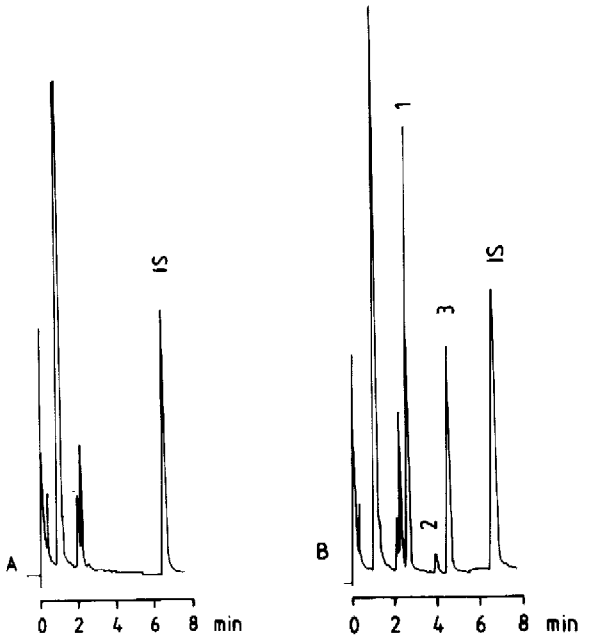

Fig. 1. Gas chromatograms of (A) blank plasma spiked with tolazoline (internal standard; 6.5 min) and (B) a sample taken from a subject containing (1) sparteine (2.9 min; 175 ng/ml), (2) 5-dehydrosparteine (4.1 min; 25.8 ng/ml), (3) 2-dehydrosparteine (4.6 min; 272 ng/ml) and (IS) tolazoline (6.5 min).

calibration graphs ($n=5$). The procedure was highly reproducible and the relative standard deviations for all compounds did not exceed 5%. The extraction of SP from plasma was approximately complete (104 ± 8%), whereas that of 5-DHS was 70 ± 5% ($n=5$). The detector response of 5-DHS was about 30% that of SP, which observation, together with the difference in extraction yield between SP and 5-DHS, was applied to determine 2- and 5-DHS concentrations in plasma. The applied extraction procedure involves fewer steps than those published by other investigators [2].

Initially splitless fluid injection was applied, with benzphetamine as an internal standard. This procedure had the shortcomings that the detection limit was unfavourable, which was partially caused by a large injection peak, and the lifetime of the column was very short owing to stripping of the stationary phase. On applying solid injection, benzphetamine had to be replaced with tolazoline, because it turned out to be extremely volatile.

Assay in urine

Typical chromatograms obtained after extraction of blank urine and a urine sample obtained from a subject participating in the study are shown in Fig. 2. Also shown is a chromatogram after borohydride reduction. The retention times were 3.7 min for SP, 5.0 min for 5-DHS, 5.5 min for 2-DHS and 7.1 min for the internal standard (tolazoline). The calibration graphs were linear in the concen-

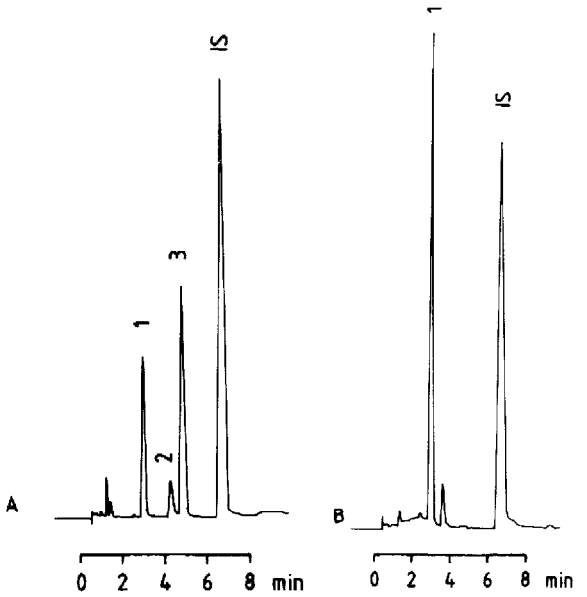

Fig. 2. Gas chromatogram (A) before reduction of (1) sparteine (3.7 min; 2.2 μg/ml), (2) 5-dehydrosparteine (5.0 min), (3) 2-dehydrosparteine (5.5 min) and (IS) tolazoline (7.1 min) in urine and (B) after reduction of the same sample with sodium borohydride: (1) sparteine (3.7 min; 7.5 μg/ml).

tration range 100–2200 ng/ml and the coefficients variations of for SP were 4% before and after reduction. The reproducibility for the compounds in urine was good and the relative standard deviations did not exceed 7%. The borohydride reduction seemed to be complete with regard to the absence of peaks at the retention times of 2- and 5-DHS in the urine from the EM after reduction, as also found by Inaba et al. [13]. DHS in urine was determined by the borohydride procedure, because this procedure is reproducible and frequently applied [12,13].

Tolazoline or benzphetamine may be applied as the internal standard in urine, but other compounds also seem to be suitable [13].

Human experiments

The plasma concentration–time curves of SP and 2- and 5-DHS obtained in an EM after intake of 50 mg of SP are shown in Fig. 3. In the PM no measurable metabolite was formed and therefore only the curve for the unchanged drug is shown in Fig. 3. Pharmacokinetic data of these subjects are given in Table I. The total plasma clearance of SP was 1172 ml/min in the EM and 210 ml/min in the PM, whereas the metabolic clearance was 605 ml/min in the EM and minimal in the PM. The plasma half-life was considerably longer in the PM (252 min) than in the EM (116 min). The renal clearance of 159 ml/min in the PM and 567 ml/min in the EM indicates that in addition to glomerular filtration presumably also tubular secretion takes place, as been suggested previously [2]. Regarding the high clearance of SP in the EM, SP cannot be regarded a low-clearance drug as has been suggested by Jacqz et al. [15].

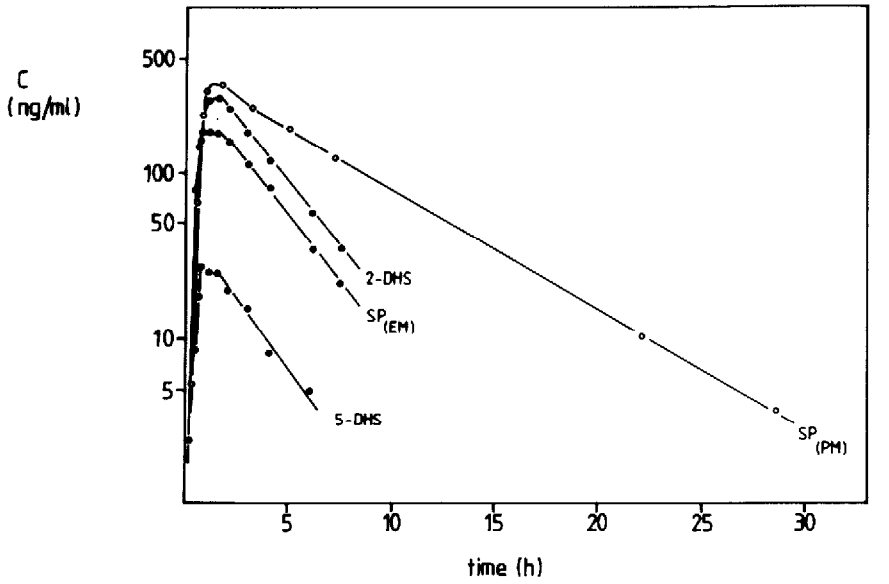

Fig. 3. Plasma concentration–time curves for sparteine, 2- and 5-dehydrosparteine in plasma after administration of 50 mg of sparteine to an extensive metabolizer (EM). The upper curve shows the sparteine profile of a poor metabolizer (PM) after intake of the same dose (no metabolites detectable).

TABLE I

PHARMACOKINETIC DATA FOR SPARTEINE (ADMINISTERED AS 50 mg OF SPARTEINE IN A COCKTAIL WITH NIFEDIPINE AND MEPHENYTOIN) IN AN EXTENSIVE METABOLIZER (EM) AND IN A POOR METABOLIZER (PM)

$Cl_{p.o.}$ = oral clearance, determined by dividing dose by area under the plasma concentration–time curve; Cl_m = metabolic clearance; $C_{\rightarrow DHS}$ = formation clearance to the sum of 2- and 5-dehydrosparteine; $SP_{0-32\,h}$ and $DHS_{0-32\,h}$ = amount of drug and metabolites excreted in the 0–32 h urine; $SP/DHS_{0-8\,h}$ = metabolic ratio as determined in the 0–8 h urine portion; $\%D_{urine}$ = percentage of the dose excreted into urine.

Subject	$Cl_{p.o.}$ (ml/min)	Cl_m (ml/min)	$Cl_{\rightarrow DHS}$ (ml/min)	Half-life (min)	$SP_{0-32\,h}$ ($\%D_{urine}$)	$DHS_{0-32\,h}$ ($\%D_{urine}$)	$SP/DHS_{0-8\,h}$
EM	1173	605	402	116	48.3	34.3	1.26
PM	210	–	–	252	75.5	–	∞

The recovery of SP in 32-h urine was 48.4% and that of DHS was 34.3% in the EM, whereas in the PM the recovery of unchanged drug in 32-h urine was 75.5%. The clearance by formation of DHS (sum of 2- and 5-DHS) in the EM was 402 ml/min.

CONCLUSION

The method developed for the determination of SP and 2- and 5-DHS in plasma and urine is sensitive, selective and accurate. The sample preparation, especially for the assay in plasma, involves fewer steps than in previously published meth-

ods. After administration of a relatively low dose of 50 mg, the elimination kinetics of SP and both metabolites can be followed for at least three times the half-life of the compounds. This makes it possible to determine kinetic parameters such as clearance and the formation of metabolites, which is of great practical value in elucidating the factors that influence the oxidation of the SP-type polymorphic drugs.

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