THE METABOLISM OF [^{14}C]-DEBRISOQUINE IN MAN

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- 1 The synthesis of [^{14}C]-debrisoquine hydrochloride and 4-hydroxy-debrisoquine sulphate is described.
- 2 The metabolic fate and excretion profile in both urine and faeces of ^{14}C -labelled debrisoquine was studied in five healthy human subjects.
- 3 Investigations showed that the drug is well-absorbed after a single oral dose of 32 mg and quantitatively eliminated from the body within three days.
- 4 4-Hydroxy-debrisoquine is the major metabolite of debrisoquine, although significant amounts of 5-, 6-, 7- and 8-hydroxy-debrisoquine are also formed.
- 5 Electron-capture gas chromatography is a useful method for measuring debrisoquine and its five hydroxylated metabolites in urine at the pg level.

Introduction

Debrisoquine was first introduced into the U.K. as an orally active antihypertensive agent in 1966 (see Athanassiadis, Cranston, Juel-Jensen & Oliver, 1966; Kitchin & Turner, 1966; Editorial, 1966). Chemically, debrisoquine is described as 3,4-dihydro-2-(1*H*)-isoquinoline-carboxamidine and is related structurally and pharmacologically to other guanidine based antihypertensives such as bethanidine and guanethidine.

Recent studies have shown that debrisoquine is metabolized in the body but that the extent to which this occurs is mainly genetically determined (Mahgoub, Idle, Dring, Lancaster & Smith, 1977). Furthermore, responsiveness to the drug is influenced by metabolic status since subjects who poorly metabolize the drug are much more sensitive to the hypotensive effects than those who extensively metabolize the drug (Idle, Mahgoub, Lancaster & Smith, 1978). Because of the important relationship between genetically determined ability to metabolize the drug and responsiveness it became important to define as fully as possible the qualitative and quantitative aspects of the metabolism of the drug in man.

Earlier studies have shown that the drug can undergo metabolism along two pathways, namely oxidation of the alicyclic and aromatic ring structures and by ring cleavage to give two acidic metabolites, namely, 2-(guanidinoethyl)benzoic acid and 2-(guanidinomethyl)phenyl acetic acid (Allen, East, Francis & Haigh, 1975; Allen, Brown & Marten, 1976; Angelo, Dring, Lancaster, Latham & Smith,

1975). In this paper we describe the qualitative and quantitative aspects of the metabolism and elimination of orally administered debrisoquine in five healthy male volunteers. Part of this work has already been presented as a preliminary communication (Angelo, Dring, Idle, Lancaster, Mahgoub & Smith, 1977).

Methods

Synthesis of 3,4-dihydro-2-(1H)-isoquinoline [^{14}C]-carboxamidine hydrochloride ([^{14}C]-debrisoquine)

[^{14}C]-Cyanamide was prepared as described by Graff, Engelman, Gillespie & Graff (1951). A solution of potassium [^{14}C]-cyanide (1.13 mg, 1 mCi) (The Radiochemical Centre, Amersham, Bucks, U.K.) and non-radioactive potassium cyanide (649 mg) in water (6.5 ml) was added over a 15 min period to a stirred and cooled (0°C) mixture of bromine (0.5 ml) and water (7.0 ml). After 15 min, the pale yellow solution was extracted with diethyl ether (3 × 10 ml) and the bulked extracts dried over anhydrous sodium sulphate for 3 h. The ethereal solution was then treated, without the removal of the sodium sulphate, with a solution of ammonia in methanol (2.9 M, 5 ml). Ammonium bromide precipitated and the mixture was then left overnight at 0°C to complete the reaction. The solids were filtered and the solvent removed in a rotary evaporator. The oily residue was dried *in vacuo* over phosphorus pentoxide for 20 h, triturated with dry ether and the small amount of ammonium bromide

that precipitated, filtered. On evaporation, the filtrate yielded 368 mg crystalline [^{14}C]-cyanamide.

The next step was a modification of the method of Wenner (1965). [^{14}C]-Cyanamide (368 mg) and 1,2,3,4-tetrahydroisoquinoline hydrochloride (1.56 g), suspended in toluene (8 ml), were then refluxed together for 6 h. After 2–3 h an extremely viscous lower phase was apparent. After cooling, and allowing to stand for 15 h, the upper toluene layer was decanted and the lower glass-like layer dissolved in ethanol (2 ml) and the solution cooled in an ice-bath. The addition of acetone (5 ml) gave a crystalline solid which was re-crystallized twice from ethanol to give 576 mg 3,4-dihydro-2-(1*H*)-isoquinoline [^{14}C]-carboxamidine hydrochloride (specific activity 0.50 $\mu\text{Ci}/\text{mg}$), m.p. 176–179°C (Wenner, 1965 quotes 179–181°C). The radiochemical yield was 28.8%, calculated on the potassium [^{14}C]-cyanide. The radiochemical purity was determined by reverse isotope dilution and paper chromatography in solvent A followed by radiochromatogram scanning and found to be >99%.

Synthesis of 4-hydroxy-3,4-dihydro-2-(1H)-isoquinoline carboxamidine sulphate (4-hydroxy-debrisoquine sulphate)

2-(1'-Hydroxy-2'-nitroethyl)-benzaldehyde lactol (2.93 g; m.p. 120–122°C dec.) was first synthesized by the method of Baer & Achmatowicz (1964).

A solution of the lactol (1.0 g) in 50% acetic acid (10 ml) was then hydrogenated for 2–3 h according to Nakao, Yura & Ito (1966) in the presence of platinum dioxide (40 mg) at a pressure of 280 kPa. The platinum dioxide was removed by filtration, the filtrate reduced to dryness *in vacuo* at 35°C and the resulting pale yellow oil washed with water (2 $\times$ 10 ml) to remove acetic acid. The residue was then taken up in the minimum volume of ethanol and treated with a slight excess of HCl in ethanol. The solution was evaporated to dryness and the residue taken up in ethanol (20 ml) and further evaporated *in vacuo* to remove any remaining trace of acetic acid. The residue was re-crystallized from ethanol to give white crystals of 4-hydroxy-1,2,3,4-tetrahydroisoquinoline hydrochloride (458 mg) (m.p. 196°C).

The final condensation step was performed by a modification of the method of Wenner (1965). A solution of 4-hydroxy-1,2,3,4-tetrahydroisoquinoline hydrochloride (458 mg) in water (10 ml) was made strongly basic by the addition of a few drops of 10 M NaOH and the milky base liberated extracted with dichloromethane (3 $\times$ 10 ml). The bulked organic extracts were dried by filtration through Whatman Phase Separating Paper and the solvent removed by evaporation *in vacuo*. The pale yellow residue (416 mg) was suspended in water (2 ml) and then mixed with a solution of *S*-methylpseudothiourea

sulphate (435 mg) in water (3 ml). The unstoppered reaction mixture was allowed to stand at room temperature in a fume cupboard for 16 h, whence crystals of 4-hydroxy-3,4-dihydro-2-(1*H*)-isoquinoline carboxamidine sulphate (4-hydroxy-debrisoquine sulphate) began to separate. These were filtered to give 68 mg of product which had m.p. 253–254°C without re-crystallization. It was characterized by comparison with an authentic sample (m.p. 253–254°C) which was the gift of Roche Products Ltd., U.K. Both samples (in KBr discs) had identical infra-red spectra (Perkin-Elmer 1579) with strong absorptions at 3.0 (O–H), 3.1 (N–H), 6.0 (C=N), 6.3 (C=N) and 9.2 (O–H) μm . The proton n.m.r. spectra (Bruker HFX 90) were also equivalent.

Synthesis of N-carbamoyl-1,2,3,4-tetrahydro-isoquinoline (debrisoquine urea analogue)

The method used was essentially that of Bamberger & Dieckmann (1893). 1,2,3,4-tetrahydroisoquinoline hydrochloride (1.68 g) and potassium cyanate (0.81 g) were each dissolved separately in the minimum quantities of water. The two solutions were then mixed and allowed to stand at room temperature. Shiny white leaflets separated which were filtered and dried *in vacuo*. *N*-Carbamoyl-1,2,3,4-tetrahydro-isoquinoline (1.4 g) had m.p. 169°C (Bamberger & Dieckmann quote 169°C).

Other reference compounds

3,4-Dihydro-2-(1*H*)-isoquinoline carboxamidine (debrisoquine sulphate; Declinax) (m.p. 274–276°C) and its phenolic derivatives, namely, 5- (m.p. 298–300°C), 6- (m.p. not determined), 7- (m.p. 303°C dec.) and 8-hydroxy-debrisoquine (m.p. 302–304°C) were a gift of Roche Products Ltd, U.K. 3,4-Dihydro-2-(1*H*)-isoquinoline carboxamidinoxime-*p*-toluene sulphonate (*N*-hydroxy-debrisoquine m.p. 112–114°C) was the gift of Sterling-Winthrop Research Institute, Rensselaer, N.Y., U.S.A.

Human investigations

For the ^{14}C studies, healthy normotensive adult male volunteers were given orally a mixture of debrisoquine sulphate (30 mg) and [^{14}C]-debrisoquine hydrochloride (10 mg; 5 μCi) (total drug equivalent to 31.7 mg free base) as a solution in water or contained in gelatine capsules. In four investigations, urine was collected at 1, 2, 3, 4, 6, 10, 24, 48 and 72 h and faeces at 24, 48 and 72 h post-dosing. In three other investigations, urine was simply collected up to 24 h and the samples bulked with no collection of stools.

Determination of ^{14}C in urine and preliminary separation of metabolites

Radioactivity in urine fractions was determined by scintillation spectrometry (Packard models 3214, 3320 and 3385) using an aliquot of urine (0.1–1.0 ml) in a dioxane-based scintillant. Primary separation of ^{14}C -labelled urinary metabolites was effected by paper chromatography. In certain cases, the urine samples were too dilute for chromatographic analysis and were concentrated as follows: the urine was initially concentrated by lyophilization and the solid residue leached with methanol (20–100 ml). The methanol extract was then reduced to 1–2 ml *in vacuo* and stored overnight at -20°C to precipitate urea which was removed by filtration. The clear solution was evaporated to dryness in a rotary evaporator at 30°C and the residue re-dissolved in water. This aqueous fraction could then be applied to a column of Amberlite XAD-2 resin which had been pre-washed successively with acetone, methanol and water and then employed according to Mulé, Bastos, Jukofsky & Saffer (1971). The methanolic eluate from this procedure contained over 90% of the applied ^{14}C . It was evaporated to dryness and the residue dissolved in the minimum quantity of methanol and the solution used for paper chromatography. The concentrated metabolite solutions were chromatographed on Whatman 3 mm paper and developed in solvent systems A and B. Prior to application all samples were adjusted to pH 2 with 1 M HCl as this largely prevented the phenomenon of 'double-spotting'. Reference standards dissolved in blank urine were co-chromatographed and located using the following spray reagents: (a) Sodium nitroprusside—hydrogen peroxide spray for guanidines (Hofmann & Wünsch, 1958); a mixture of 5% aqueous sodium nitroprusside (2 ml), 3% aqueous sodium hydroxide (1 ml), 3%

aqueous hydrogen peroxide (5 ml) and water (20 ml). Guanidines gave coloured spots on a yellow background. (b) Diazotized 4-nitroaniline for phenols: 4-nitroaniline (0.25 g) was dissolved in 1 M HCl (25 ml) and the solution diluted with ethanol (25 ml). Sodium nitrite (0.1 g) was added to 10 ml of this solution and the chromatograms sprayed. After drying, the chromatograms were re-sprayed with 0.5 M NaOH in ethanol (Wickström & Salvesen, 1952). Phenolic metabolites (5-, 6-, 7- and 8-hydroxy-debrisoquine) gave red spots. (c) 4-Dimethylamino-benzaldehyde (DMAB) for urea derivatives: DMAB (0.5 g) was dissolved in a mixture of 2 M HCl (50 ml) and ethanol (50 ml). *N*-Carbamoyl-1,2,3,4-tetrahydro-isquinoline gave a canary yellow spot after standing for 12 h.

The R_F values and colour reactions of debriosoquine and its derivatives are shown in Table 1.

^{14}C -Bands on the paper chromatograms were located by radiochromatogram scanning (Packard scanner, models 7200 and 7201). Further resolution, identification and quantitation of the resulting four radioactive bands occurring at R_F 0–0.14, 0.24–0.43, 0.43–0.61, and 0.62–0.81 in System A is later described.

Determination of ^{14}C in faeces

All faecal samples were homogenized in 5 vol. of water and bleached prior to counting. Faecal homogenate (20 ml) was adjusted to pH 10 with 10 M NaOH and an equal volume of hydrogen peroxide (100 vol) added. The mixture was left to stand at ambient temperature for 2 days, then neutralized. Bleached supernatant (0.5 ml) was then counted like urine in liquid scintillant. No qualitative procedures were performed on the faecal homogenates.

Table 1 Paper chromatography of debriosoquine and its derivatives

Compound	* R_F in solvent		Colour reactions with:		
	A	B	nitroprusside	diazotized 4-nitro-aniline	DMAB
Debrisoquine	0.73	0.47	Pink	None	None
4-hydroxy-debrisoquine	0.28	0.33	Pink	None	None
5-hydroxy-debrisoquine	0.58	0.25	Purple	Reddish brown	None
6-hydroxy-debrisoquine	0.57	0.22	Purple	Reddish brown	None
7-hydroxy-debrisoquine	0.57	0.22	Purple	Reddish brown	None
8-hydroxy-debrisoquine	0.58	0.22	Purple	Reddish brown	None
Debrisoquine <i>N</i> -hydroxy derivative	0.75	0.63, 0.70	Orange	None	None
Debrisoquine urea analogue	0.81	0.78	Brown	None	Yellow

Whatman 3 mm paper was used. Solvent compositions were: A: butan-2-ol: formic acid: water (100:12:10 v/v⁺) and B: propan-2-ol: ammonia (sp. gr. 0.88): water (20:1:2 v/v).

* R_F calculated from centre of the large diffuse spots.

⁺ Aged for 1 week.

Reverse isotope dilution for debrisoquine in urine

Debrisoquine sulphate (approx. 1 g accurately weighed) was added to the urine sample to be analysed (containing >150,000 d/min) and, where necessary, water added to facilitate the dissolution of the carrier. The solution was made strongly basic (pH >13) with 10M NaOH, saturated with sodium chloride and extracted with dichloromethane (3 × 2 vol). The pooled extract was dried by filtration through Whatman Phase Separating Paper and then evaporated to dryness *in vacuo*. The residue was then dissolved in the minimum volume of dry ethanol, and a slight excess of HCl in ethanol added. After adding a few drops of diethyl ether, precipitation of [¹⁴C]-debrisoquine hydrochloride occurred; the solid was filtered and re-crystallized to constant specific activity from ethanol (m.p. 179–180°C).

Isolation of metabolite fractions

Concentrated methanolic eluates from XAD-2 columns (see above) were resolved into four major ¹⁴C fractions by paper chromatography in system A as described. The chromatographic bands were cut out and eluted separately by submergence in beakers containing methanol (100 ml) for up to 2 h. The methanol fractions were reduced to a small volume (<1 ml) *in vacuo* and then further purified by t.l.c. using silica gel 60F₂₅₄ plates (E. Merck, Darmstadt, W. Germany) which had been washed with methanol.

After location of radioactivity by radiochromatogram scanning, the ¹⁴C areas were scraped off the plate and eluted with methanol. The metabolites in the methanolic eluates were subsequently identified by gas-chromatography and by gas-chromatography-mass spectrometry (*vide infra*).

Gas-chromatographic methods

Gas chromatography was utilized both to identify and measure the various metabolites of debrisoquine in human urine. Two methods were applied: in the first, flame-ionization detection (f.i.d.) was used to identify debrisoquine and 4-hydroxydebrisoquine; in the second, electron-capture detection (e.c.d.) was used to both identify and quantitate debrisoquine and its hydroxylated metabolites, namely, 4-, 5-, 6-, 7- and 8-hydroxy-debrisoquine.

Flame-ionization detection (f.i.d.)

Reference compounds, freeze-dried urinary extracts and isolated metabolite fractions in methanol were derivatized with acetylacetone according to Shemyakin, Ouchinnikov, Vinogradova, Feigina, Kiryushkin, Aldanuva, Alakhov, Lipkin & Rosinov (1967) and

Vetter-Diechtl, Vetter, Richter & Biemann (1968). This reaction converts debrisoquine and its derivatives to dimethylpyrimidine derivatives which are readily extracted by organic solvents and are volatile and therefore more amenable to gas-chromatography than their polar amidine precursors. To a solution of sample (100 µg) in 50% saturated aqueous sodium bicarbonate (0.2 ml) and methanol (0.8 ml) was added freshly-distilled acetylacetone (0.2 ml). After heating in a capped vial at 50°C for 48 h, water (4.8 ml) was added. The pH was adjusted to 8.5 with 2 M NaOH and the mixture extracted with chloroform (3 × 12 ml). Bulk extracts were reduced to dryness *in vacuo* and the residue dissolved in pyridine (100 µl) for injection into the gas chromatograph or for further derivatization of phenolic groupings, with either *N,O*-bis-(trimethylsilyl)-acetamide (BSA; Phase Separations Ltd, Queensferry, Flintshire, U.K.) or *N,O*-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) + 1% trimethylchlorosilane (TMCS; Pierce Chemicals Co., Rockford, Ill., U.S.A.). Samples (1–2 µl) were injected into a Hewlett Packard F&M Scientific 402 gas chromatograph fitted with a 3% OV-225 column (support phase: Chromosorb W, 100–120 mesh; length: 1.22 m; 2 mm i.d.) and a flame-ionization detector; carrier gas was nitrogen with a flow rate of 28 ml min⁻¹. Chromatograms were run isothermally at 200°C with the flame-ionization detector maintained at 250°C.

Electron-capture detection

Debrisoquine and its hydroxylated metabolites in urine were converted to their bis-(trifluoromethyl)-pyrimidine products by reaction with hexafluoroacetylacetone (HFAA) according to Erdmansky & Goehl (1975). To urine (100 µl) in a screw-cap septum vial was added 100 µl water containing 7-methoxy-guanoxan (10 µg) (the gift of Pfizer Ltd, Sandwich, Kent, U.K.) as internal standard, 1 M sodium bicarbonate (100 µl), hexafluoroacetylacetone (100 µl) and re-distilled benzene (1 ml). The reaction vial was heated in an aluminium heating block at 100°C for 1 h. The vial was then cooled and 3 M NaOH (5 ml) added to hydrolyse excess HFAA. The sample was vortexed, centrifuged and a portion of the benzene phase (1 µl) injected into a Becker 417 gas chromatograph fitted with a tritium-scandium electron-capture detector (75 mCi) and a 3% OV-1 column (support phase: Chromosorb W. HP, 80–100 mesh; length: 1.83 m; 3 mm i.d.). Carrier gas was 5% argon in methane with a flow rate of 60 ml min⁻¹. Chromatograms were run isothermally at 195°C with the electron-capture detector stabilized at 235°C. Because this type of detector has a linear response only over a narrow concentration range, for debrisoquine, about 1–100 pg on column, many samples had to be diluted and re-derivatized to bring them into the

Figure 1 Calibration curves for debrisoquine (○) and 4-hydroxy-debrisoquine (●).

range of the calibration curve of concentration plotted against ratio of peak heights debrisoquine: internal standard. This assay measured debrisoquine and 4-hydroxy-debrisoquine. To quantitate the phenolic metabolites, a solution of BSA (10% in benzene; 50 μ l) was added to an equal volume of the benzene layer from the derivatization reaction and the mixture shaken. An aliquot (1 μ l) of this solution was injected onto the column and the trimethylsilyl ethers of the phenols resolved with a flow rate of 30 ml min⁻¹ and a column temperature of 160°C. The retention times of the compounds relative to 7-methoxyguanosine were: debrisoquine, 0.19; 4-hydroxy-debrisoquine, 0.41; 5-hydroxy-debrisoquine, 0.59; 6-hydroxy-debrisoquine, 0.75; 7-hydroxy-debrisoquine, 0.67; 8-hydroxy-

Figure 2 Calibration curves for 5- (▽), 6- (■), 7- (□) and 8-hydroxy-debrisoquine (▲).

debrisoquine, 0.54. Calibration curves for debrisoquine and its five hydroxylated metabolites are shown in Figures 1 and 2. The standard deviation in peak height ratio across the full range of concentrations was less than 5% for all six curves.

Gas chromatography—mass spectrometry

Samples of lyophilized human urine derivatized with acetylacetone as described were analysed by gas chromatography—mass spectrometry (g.c.m.s.). A Varian MAT 311A mass spectrometer was used interfaced with a Varian Aerograph 1400 using a column of 3% OV-225 on Chromosorb W and programmed from 200°C to 270°C at 6°C min⁻¹. Helium was employed as carrier gas. Mass spectra were acquired on all peaks in the chromatogram and compared with those obtained from standards run similarly.

Results

Five human subjects were each given [¹⁴C]-debrisoquine orally at a dose equivalent to 32 mg of the free base. Two of these volunteers were similarly re-investigated some months later. The data derived from these seven investigations is summarized in Table 2. The drug was well-absorbed, 72.0 $\pm$ 5.3% ($\pm$ s.d.) of the ¹⁴C being recovered in the urine within 24 h. In four of the seven investigations, urine and faeces were collected up to 72 h. The second and third day urines contained 8.9 $\pm$ 3.2 and 2.3 $\pm$ 1.4% of the dose, and the first, second and third day faeces contained 4.6 $\pm$ 7.2, 2.8 $\pm$ 1.2 and 0.9 $\pm$ 1.3% of the dose respectively. The total radioactivity excreted in the urine and faeces of four subjects in 3 days was thus 93.8 $\pm$ 5.6% of the administered dose.

Radiochromatographic investigation of the 0–24 h urine revealed four ¹⁴C bands of R_F 0–0.12, 0.25–0.43, 0.43–0.61, 0.62–0.81 in system A. A typical radiochromatogram scan of a paper run in system A is shown in Figure 3. From their position on the chromatograms and colour reactions to the

Figure 3 Typical radiochromatogram scan of urinary excretion products of [¹⁴C]-debrisoquine indicating at least four major radiolabelled entities.

Table 2 Metabolism and excretion of a single oral dose of 32 mg debrisoquine in five human subjects

	Subject				
	1	2	3	4	5
Metabolite (% of dose in 0-24 h urine)					
Debrisoquine ¹	54.8 (54.0) ²	23.2 (23.0) ²	25.8 (27.8) ³	18.8 (18.0) ²	23.0 (26.5) ³
4-Hydroxy-debrisoquine ¹	5.4	16.7	29.1 (37.0) ³	24.2	33.4 (38.8) ³
5-Hydroxy-debrisoquine ³	—	—	1.3	—	2.3
6-Hydroxy-debrisoquine ³	—	—	1.0	—	3.6
7-Hydroxy-debrisoquine ³	—	—	1.9	—	6.3
8-Hydroxy-debrisoquine ³	—	—	0.6	—	1.5
Total phenols ³	—	—	4.8	—	13.7
Polar metabolite fraction	15.1	9.1	ND	5.8	ND
% of dose recovered in					
0-24 h urine ⁴	69.0	74.2	66.8 (69.5) ³	75.6	77.6
24-48 h urine ⁴	11.5	11.8	—	6.6	5.6
48-72 h urine ⁴	3.9	0.9	—	2.8	1.4
0-24 h faeces ⁴	3.1	ND	—	15.1	ND
24-48 h faeces ⁴	3.7	2.1	—	1.5	3.9
48-72 h faeces ⁴	0.7	ND	—	0.2	2.8
0-72 h urine + faeces ⁴	92.9	89.0	—	101.8	91.3

¹ determined by radiochromatography (paper)² determined by reverse isotope dilution³ determined by electron-capture gas chromatography⁴ determined by scintillation spectrometry

— means not analysed

ND means not detected.

Figure 4 Mass spectrum of debriisoquine isolated from human urine, derivatized with acetylacetone (see text).

locating sprays (see Table 1), the bands were initially identified as debriisoquine (R_F 0.62–0.81), 4-hydroxy-debriisoquine (0.25–0.43), a band corresponding to the phenolic metabolites 5-, 6-, 7- and 8-hydroxy-debriisoquine (0.43–0.61) which were unresolved on the paper chromatograms and a polar ^{14}C band (0–0.12) which did not correspond to any of the reference compounds. Neither the *N*-hydroxylated metabolite nor the urea analogue was detected by paper chromatography as urinary metabolites of debriisoquine. Confirmation and measurement of the various metabolites of debriisoquine is discussed hereafter.

Debriisoquine

The presence of the unchanged drug in urine was confirmed by gas chromatography on OV-225 (f.i. detection) and OV-1 (e.c. detection) and by g.c.m.s. In all three systems a peak (R_t 4.9 min on OV-225) occurred in the chromatogram after injection of derivatized urinary extracts at the same retention time as debriisoquine (R_t 4.9 min on OV-225). Furthermore, the mass spectrum obtained from this peak was identical for authentic debriisoquine (Figure 4). Urinary debriisoquine was quantitated by three procedures, good correspondence being found for the

Figure 5 Mass spectrum of 4-hydroxy-debriisoquine isolated from human urine, derivatized with acetylacetone (see text).

three modes of assay (see Table 2). Firstly, the drug was quantitated by scintillation counting of the ^{14}C areas on paper chromatograms of urine samples, corresponding to debriisoquine (band of R_F 0.62–0.81 in solvent A). Secondly, by reverse isotope dilution and thirdly by quantitative e.c.g.c. Unchanged drug was excreted in large and variable amounts by the five subjects studied; 30.4 ± 14.9 (range 18.6–54.8) % of the dose of debriisoquine was found to be excreted unchanged in the 0–24 h urine.

4-Hydroxy-debriisoquine

The presence of 4-hydroxy-debriisoquine in urine and in eluates of paper chromatograms of urine samples was also confirmed by gas-chromatography and g.c.m.s. Both urine samples and eluates of metabolites derivatized with acetylacetone showed a peak (R_t 7.3 min) corresponding to 4-hydroxy-debriisoquine (R_t 7.4 min) by gas chromatography on OV-225. The mass spectrum taken from this peak was also identical to that from the reference sample (Figure 5). 4-Hydroxy-debriisoquine was quantitated by paper chromatography followed by radiochromatogram scanning and e.c.g.c. A poor correspondence was observed between two assays, the radiochromatographic assay consistently giving lower values than the g.c. method (see Table 2). Because 4-hydroxy-debriisoquine was detected in the eluate of phenolic metabolites by e.c.g.c., it is obvious that this compound does not move as a single spot in this paper chromatographic system, thereby giving low estimates, when estimated by quantitative radiochromatogram scanning. The excretion of this metabolite as shown by radiochromatography shows a large inter-individual variation, $21.1 \pm 9.5\%$ (5.4–33.4) of the dose excreted in the 0–24 h urine as 4-hydroxy-debriisoquine.

5-, 6-, 7- and 8-Hydroxy-debriisoquine

The ^{14}C band which eluted at R_F 0.43–0.61 on paper in system A, corresponded to these phenolic metabolites by R_F values and also gave a strong positive reaction to diazotized 4-nitroaniline spray, indicating the presence of phenols. When this band was isolated, purified as described and derivatized with hexafluoroacetylacetone followed by BSA, peaks corresponding to debriisoquine, 4-, 5-, 6-, 7- and 8-hydroxy-debriisoquine were seen (Figure 6). None of these peaks occurred in derivatized blank urine or in reagent blanks run on the gas chromatograph and were thus debriisoquine-related. This separation was used as the basis of an assay to quantitate 5-, 6-, 7- and 8-hydroxy-debriisoquine. When the appropriate ^{14}C eluate was derivatized with acetylacetone and run on OV-225, peaks corresponding to all four phenols were also seen. The e.c.g.c. method affords a method

Figure 6 Gas-chromatographic separation of the phenolic metabolites of debrisoquine (see text). a) standards of the metabolites, b) metabolites extracted from human urine. i.s. 7-methoxy-guanoxan.

of resolving and measuring the small amounts of these phenolic metabolites in urine. In the three urines which were analysed by this method, the phenols were relatively minor metabolites, comprising in total $7.1 \pm 5.8\%$ (2.7–13.7) of the dose, the 7-hydroxylated compound being the most abundant phenol found. Incubations of urine with β -glucuronidase (Ketodase, Warner-Chilcott, Eastleigh, Hants., U.K. at pH 5) or sulphatase (Sigma Chemical Co., Kingston-upon-Thames, U.K. at pH 5) did not increase the amounts of debrisoquine, 4-hydroxy-debrisoquine or the phenolic metabolites indicating that these are excreted unconjugated in man.

The polar ^{14}C material (R_F 0–0.12 in system A) was not further investigated in man, although this metabolite fraction in the rat seems to be a composite of debrisoquine and conjugated 4-hydroxy-debrisoquine, 6-hydroxy-debrisoquine, a dihydroxylated metabolite and two carboxylic acids arising from oxidative ring scission (M.M. Angelo, L.G. Dring and R.L. Smith, unpublished observation). This polar band accounted for $3.9 \pm 4.7\%$ ($\pm$ s.d.) (0–11.2) of the dose.

Figure 7 Pathways of oxidation of debrisoquine (D) in man.

Discussion

Debrisoquine, a strongly basic guanidine of pK_a 12.5, was well-absorbed when given orally and rapidly excreted by man. Greater than 70% of a dose of 32 mg was found in the urine within 24 h and almost complete elimination (89–102%) by urinary and faecal excretion occurred within 3 days. Debrisoquine and five monohydroxylated metabolites namely, 4-, 5-, 6-, 7- and 8-hydroxy-debrisoquine were identified and quantitated in the urine (see Figure 7). Except for a small amount of highly polar derivatives ($3.9 \pm 4.7\%$, $n=7$), these metabolites accounted for all the ^{14}C eliminated in the first-day urine. Therefore, the two carboxylic acids which have been described by Allen *et al.* (1975, 1976), as urinary metabolites of debrisoquine must be minor metabolites comprising part of the polar unidentified fraction (*vide supra*).

Of the monohydroxylated metabolites, 4-hydroxy-debrisoquine is clearly the major product, but its production is prone to a large inter-individual variation. By contrast, the four phenolic metabolites individually compose much smaller fractions of the excreted dose and show less inter-subject variability. Inter-individual variations in the extent of formation of 4-hydroxy-debrisoquine have subsequently been found to be genetic in origin (Mahgoub *et al.*, 1977). The major metabolic reaction of debrisoquine *in vivo* is hydroxylation of the saturated ring system in the 4-position. It is of interest that this alicyclic carbon position is more susceptible to metabolic oxidation than the aromatic carbon centres. The alicyclic hydroxylation of debrisoquine in the 4-position introduces a chiral centre into the molecule so that 4-hydroxy-debrisoquine could exist in the R or S forms as well as the racemate. The nature of the hydroxylation of debrisoquine *in vivo* has not yet been

ascertained but quite probably it involves a stereo-specific mechanism, giving rise to all or a preponderance of one of the enantiomers as is known to occur in the hydroxylation of other compounds such as ethylbenzene (McMahon, Sullivan, Craig & Pereira, 1969) and indan (Billings, Sullivan & McMahon, 1970) (for review, Jenner & Testa, 1973).

Apparently, the guanidine moiety is metabolically stable in man. Neither the urea analogue nor the *N*-hydroxylated metabolite was detected. This is in accord with the general observation that with guanidine-based drugs such as guanoxan (Jack, Stenlake & Templeton, 1972), guanethidine (McMartin & Simpson, 1971), phenformin (Beckmann, 1968) and amiloride (Weiss, Hersey, Dujovne & Bianchine, 1969) the guanidine nitrogens are metabolically inert.

It is interesting to propose that the alicyclic hydroxylation of debrisoquine in the 4-position and the aromatic hydroxylation in the 5-, 6-, 7- and 8-positions (see Figure 7) may be effected by quite separate mechanisms. Classically, aromatic hydroxylation to yield phenols is considered to be mediated via arene oxide formation (see Jerina & Daly, 1977 for a review) although there are singular exceptions such as the 6- and 7-hydroxylation of butamoxane [2-(butylaminomethyl)-1,4-benzodioxane] by rat liver microsomes which proceeds by the direct insertion of oxygen without either an arene oxide or dihydrodiol intermediate (Murphy, Bernstein & McMahon, 1974).

The mechanism of alicyclic *C*-oxidation, however, remains largely an enigma; it is hard to visualize the involvement of such intermediates as arene oxides with saturated ring systems. One proposal would be that the aromatic and alicyclic hydroxylations involve different forms of cytochrome P450. Biphenyl, bromobenzene, *n*-hexane, benz[a]pyrene and testosterone are well documented examples of molecules where hydroxylation occurs at different sites in the structure, one mechanism mediated *in vitro* by a phenobarbitone-inducible (P450) and the other by a 3-methyl-cholanthrene-inducible (P448) cytochrome system (Atlas & Nebert, 1977).

Debrisoquine is a compound which produces five monohydroxylated metabolites in man which can readily be quantitated by electron-capture gas chromatography at the pg level: It remains to be seen whether or not debrisoquine will prove useful as an *in vivo* probe for differentiating possible multiple forms of cytochrome P450 and individual variations in their occurrence.

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