Metabolism of thymoxamine: identification of metabolites in rat

C. FENIOU*, B. NEAU, G. PRAT, C. CHEZE†, F. FAURAN, J. ROQUEBERT†

Sarget Research Department, Avenue J. F. Kennedy, 33701 Merignac, France

Thymoxamine hydrochloride administered by mouth to rats at 25 or 100 mg kg^{-1} was excreted in the urine as the deacetyl and N-demethyl-deacetyl metabolites. These were completely sulpho- and glucuronoconjugated at 25 mg kg^{-1} but only partially so at the higher dose. Thymoxamine deacetylation in vitro is catalysed by plasma and hepatic cytosol esterases and the deacetyl metabolite undergoes N-demethylation catalysed by the cytochrome P 450 hepatic microsome mixed function monooxygenase system. Because of the rapidity of the deacetylation it is concluded that thymoxamine is a prodrug leading in vivo to the active deacetyl thymoxamine.

Thymoxamine, 4[2-dimethylamino) ethoxy]-2-methyl-5-(1-methyl-ethyl) phenol acetate, is a competitive α-blocking drug (Birmingham & Szolcsanyi 1965) without β-blocking activity (Foster 1966). Recently Drew (1976) has shown that the drug acts selectively at the post synaptic α-receptor.

There appears to be little information on the metabolism of thymoxamine. Arbab & Turner (1971) measuring plasma concentrations, suggested that deacetylation could be a metabolic route, but they were not able to show whether the deacetyl thymoxamine detected was formed during the extraction process. One of us (J.R.) favoured metabolic deacetylation and suggested the existence of another metabolite of non-established structure.

We have now examined the metabolism of thymoxamine in the rat to determine whether the drug itself and/or one of its metabolites is the active form of the drug.

MATERIAL AND METHODS

Animals
Male rats of Wistar strain, 270 to 310 g, were used. For the study of urinary metabolites, each treated rat received one oral dose of either 25 or 100 mg kg^{-1} thymoxamine hydrochloride in solution in a 6% suspension of arabic gum; control rats received vehicle.

Collection of urine
Rats were housed in twos in metabolic cages for at least 18 h before drug administration. Urines were collected into tubes containing 1 ml of toluene, 7,

24, 31, 48, 72 and 96 h after drug administration. After removal of toluene, urine was stored at −18 °C.

Extraction process
To extract the drug, ammonia 1M was added to pH 9 and the urine was shaken with chloroform before and after hydrolysis (see below) of sulpho- and glucuronoconjugated products. The classical liquid-liquid extraction process was sometimes replaced by a technique using Extrelut prepacked columns (Merck). The columns were filled with a large pore kieselguhr of granular structure and high pore volume. 20 ml aqueous phase (urine or solution resulting from enzymatic hydrolysis) was applied to the support material and distributed as stationary phase on the matrix and the column eluted with successive volumes of chloroform which were then combined and taken to dryness under vacuum; the residues were then dissolved in 0·2 ml ethanol.

Hydrolysis of sulpho- and glucuronoconjugated forms
Three comparative tests were performed: samples of 5 ml urine from treated rats were incubated at 37 °C for 6 h in 25 ml acetoacetate buffer at pH 5 with 0·1 ml saline, or 0·1 ml crude β-glucuronidase from *Helix pomatia* (Sigma) with β-glucuronidase (98900 Fishman units ml^{-1}) and arylsulfatase (1550000 Roy units ml^{-1} at pH 5·0) activities and purified β-glucuronidase (IBF) (70000 Fishman units ml^{-1}) from *Helix pomatia* without arylsulfatase activity.

Thin layer chromatography (t.l.c.)
Chromatograms on silica gel plates F 254 (Merck) (thickness 0·5 mm) were developed mainly with benzene–methanol (60:40 v/v) and revealed in u.v. or with a reagent either specific for amine function

(potassium iodoplatinate) or specific for phenol function (Prussian blue). We sometimes used for elution benzene–methanol–ammonia (50:50:0·5 v/v) or cyclohexane–toluene–diethylamine (75:15:10 v/v) systems.

For preparative chromatograms, silica gel plates F 254 (thickness 2 mm) were developed with benzene–methanol (60:40 v/v). The u.v. quenching zones were cut out and the corresponding compounds eluted with methanol.

Gas chromatography (g.c.) and gas chromatography-mass spectrometry (g.c.-m.s.) coupling

Gas chromatographs were recorded with a Varian 2400 apparatus working with two flame ionization detectors. Samples collected by preparative t.l.c. were analysed on a 1·80 m × 2 mm column filled with a 3·8% OV17 silicone phase on 100–120 mesh Chromosorb WHP (injector temperature 280 °C, detector temperature 280 °C, column temperature 210 °C, carrier gas nitrogen, at flow-rate 25 ml mm⁻¹. This technique enabled us to detect either 10 mg of deacetylthymoxamine or thymoxamine litre⁻¹ urine or 100 mg of demethyldeacetyl thymoxamine litre⁻¹ urine. A v.g. Micromass 16 F low resolution with a data treatment system type 20–40 was used for the g.c.-m.s. coupling.

Metabolism in liver fractions

Isolation of liver fraction (Bartosek et al 1974). Rat liver was perfused with saline and then homogenized with a 0·15 M KCl − 0·05 M phosphate buffer pH 7·4. The homogenate was centrifuged at 9000 g for 20 min to separate mitochondria. The supernatant constituted the cytosol fraction (1·5 ml cytosol fraction corresponding to 1 g liver). After washing, the pellet was resuspended and centrifuged in the same conditions. The pellet 105 000 g was then homogenized with phosphate buffer to obtain the microsomal fraction (1 ml microsomal fraction corresponding to 0·5 g liver and containing ca 10 mg protein).

Thymoxamine hydrochloride hydrolysis by cytosol fraction. A solution of 25 μg ml⁻¹ of the drug in the cytosol fraction was incubated at 37 °C for 2 to 20 min and then extracted and analysed by t.l.c. as described above.

Deacetyl thymoxamine treatment with microsomal fraction. A 6 ml solution containing 1 ml cytosol fraction, 1 ml phosphate buffer 0·05 M, pH 7,4, 2 ml microsomal fraction, glucose 6-phosphate 15 μmol, MgCl₂ 40 μmol, nicotinamide 50 μmol, deaceytl

thymoxamine hydrochloride 12 μmol, was incubated for 2 h at 37 °C with constant shaking, according to Albrecht et al (1973), and then extracted and analysed by t.l.c. as described above.

Materials

Thymoxamine hydrochloride was provided by Dedieu Laboratories. Deacetyl- and demethyl-deacetyl thymoxamine hydrochlorides were synthesized as described by Buzas et al (1959) and Gödecke (1972) respectively. All chemicals were examined by ¹H n.m.r. and m.s.

RESULTS

After the 25 and 100 mg kg⁻¹ doses two urinary metabolites I and II were seen on t.l.c. plates but thymoxamine was never detected. For the first urinary pool (0–7 h) only metabolite I was seen. The second metabolite appeared in the 7–24 h urines. The only difference between the two doses was that metabolite I was hardly detectable after 31 h for the lower dose but was detectable up to 96 h after the high dose.

The three assays carried out with and without crude (i.e. containing an arylsulphatase) or purified glucuronidase showed that after the 25 mg kg⁻¹ dose the two metabolites were completely glucurono- and sulphoconjugated whereas at the higher dose, some drug was excreted as its free metabolites.

The chromatographic properties of the metabolites I and II on comparison with reference substances led us to assume that the two compounds were respectively deacetyl thymoxamine (4-dimethyl-aminoethoxy-5-isopropyl-2-methylphenol) and N-demethyldeactyl thymoxamine (5-isopropyl-2-methyl-4-methylaminoethoxyphenol) (Table 1). Both structures were confirmed by g.c.-m.s. coupling. Their mass spectra contained 3 characteristic peaks corresponding to the fragmentation scheme of the reference products indicated in scheme 1. These peaks are first the molecular peak and then two others corresponding to fragments related to the aminoalkyl chain. Because of the difference of stability between the ammonium ions $CH_2 = \overset{+}{N}HCH_3$ and $CH_2 = \overset{+}{N} (CH_3)_2$ the base peak in the mass spectra of deacetyl thymoxamine is related to $CH_2 = \overset{+}{N} (CH_3)_2$ whereas in the mass spectra of the demethyldeacetyl compound the base peak is related to $[CH_2–CH_2–NHCH_3]^+$. Since the chromatographic properties (R_F in three solvent systems, Rt (Table 1) and m.s. of the metabolites I and II) were respectively identical to those of deacetyl- and N-demethylde-
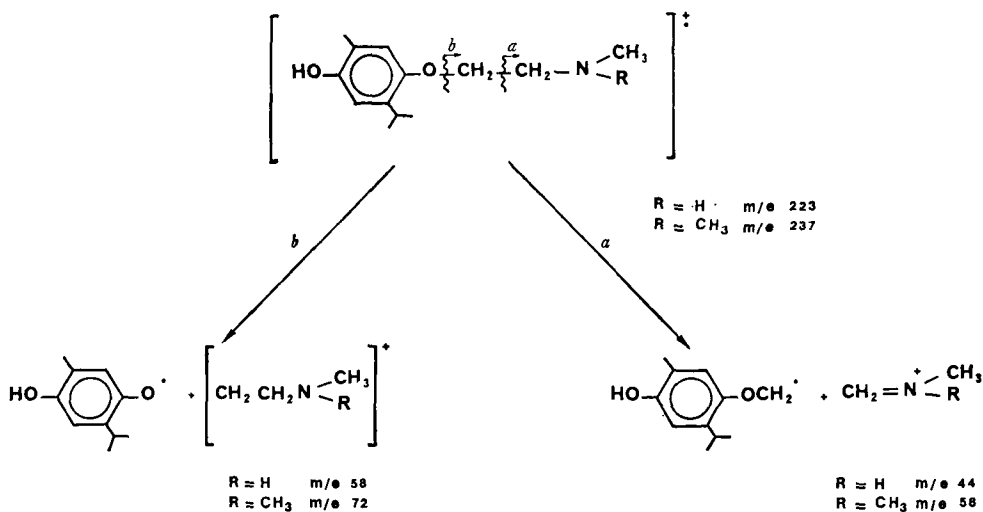
Scheme 1. Metabolism and urinary excretion of thymoxamine.

Table 1. Chromatographic R_F for three systems of solvent: A benzene–methanol 60:40 (v/v), B benzene–methanol–ammonia 50:50:0·5 (v/v), C cyclohexane–toluene–diethylamine 75:15:10 (v/v). Gas chromatography retention time Rt (column (1·80 m × 2 mm) packed with 3·8% OV17 on 100–120 mesh Chromosorb WHP, column temperature 210 °C (isothermal), nitrogen flow-rate 25 ml mm⁻¹).

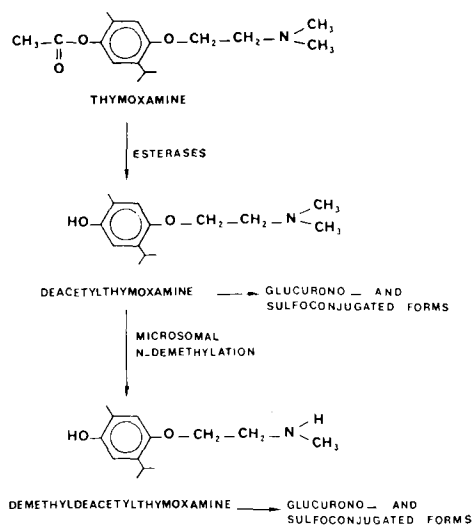| Products	R_F Solvent systems			Rt (mn)
	A	B	C	
Thymoxamine	0·38	0·55	0·19	6·20
Deacetylthymoxamine (Metabolite I)	0·31	0·54	0·10	5·20
Demethyldeacetylthymoxamine (Metabolite II)	0·11	0·31	0·05	5·90

acetyl thymoxamine we conclude that they are identical with the reference compounds.

The treatment of a thymoxamine base solution by making alkaline to pH 9 and extracting with chloroform was proof that the drug was not hydrolysed in these conditions.

In vitro thymoxamine quickly underwent hydrolysis by either plasma or cytosol esterases since after an incubation for only 2 min the drug could no longer be detected on t.l.c. plates. After an incubation of deacetyl thymoxamine with microsomal fraction t.l.c. showed besides the deacetyl spot, a new spot assigned to the N-demethylation product.

DISCUSSION

The metabolism of thymoxamine after a single oral dose in the rat involves deacetylation and N-demethylation. In vitro, deacetylation may be catalysed by the plasma or cytosol esterases and the N-demethylation by the hepatic microsome cytochrome P 450 mixed function monooxygenase system. The two phenolic compounds resulting from these reactions are then conjugated partially or completely depending on the dose used. The urinary metabolic pattern is shown in scheme 2. The deacetylation reaction is rapid since in vitro after 2 min of incubation, the drug is no longer detectable. But N-demethylation is much slower since the N-demethyldeacetyl com-

Scheme 2

pound is not present in urine until after 7 h and N-demethyl thymoxamine has never been identified in our experimental conditions. The speed of deacetylation indicates that it occurs soon after drug absorption and is certainly catalysed by esterases located in the plasma or in mucosa such as the gastric mucosa hydrolases identified recently for acetylsalicylic acid deacetylation (Spenney 1978). Since the N-demethylated product appears in urine only after the first 7 h, the circulating form of thymoxamine is deacetylated compound. This is corroborated by the findings that the deacetylated product in vitro in human isolated smooth muscle (Arbab et al 1973), and rat isolated vas deferens (Creuzet et al 1980), and in vivo in the cat (Credner & Graebner 1967: Creuzet et al 1980) and the dog (Creuzet et al 1980) has almost the same α-blocking action as thymoxamine itself. But the demethyldeacetyl compound is much less active in vitro and even inactive in vivo (Creuzet et al 1980). So there is evidence that thymoxamine is a prodrug leading in vivo to the active deacetyl form.

Acknowledgements

REFERENCES

Albrecht, R., Pellissier, M. A., Manchon, Ph., Rospars, J. P. (1973) C. R. Soc. Biol. (Paris) 167: 1552–1557

Arbab, A. G., Turner, P. (1971) J. Pharm. Pharmacol. 23: 719–721

Arbab, A. G., Turner, P., Variava Dinaz M. (1973) Br. J. Pharmacol. 47: 677P–678P

Bartosek, I., Guaitani, A., Garattini, S., Simonazzi, I. (1974) Biochem. Pharmacol. 23: 599–605

Birmingham, A. T., Szolcsányi, J. (1965) J. Pharm. Pharmacol. 17: 449–458

Buzas, A., Teste, J., Frossard, J. (1959) Bull. Soc. Chim. France 839–849

Credner, K., Graebner, R. (1967) Arzneim.-Forsch. 17: 305–309

Creuzet, M. H., Prat, G., Malek, A., Fauran, F., Roquebert, J. (1980) in the press

Drew, G. M. (1976) Eur. J. Pharmacol. 36: 313–320

Foster, R. W. (1966) J. Pharm. Pharmacol. 18: 1–12

Gödecke, A. G. (1972) Ger. Patent n° 2238260, 3rd August

Spenney, J. G. (1978) Am. J. Physiol. 234: 606–610