J. Pharm. Pharmacol. 1986, 38: 506–509
Received December 6, 1985

Chromatographic separation of thioridazine sulphoxide and *N*-oxide diastereoisomers: identification as metabolites in the rat

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(±)-Thioridazine and (±)-desmethylthioridazine have been oxidized to produce a number of chiral sulphoxide and amine oxide compounds. Diastereoisomeric isomers were separated by thin-layer and high performance liquid chromatography. Thioridazine-5-sulphoxide, *N*-desmethylthioridazine-5-sulphoxide and thioridazine-*N*-oxide diastereoisomers were found to be thioridazine metabolites following dosing in rats or after in-vitro incubation with rat liver homogenate.

In the synthesis of thioridazine (Bourquin et al 1958), two methods of obtaining (+)- and (−)-thioridazine enantiomers were described; one by fractional crystallization of (±)-thioridazine di-(*p*-toluyl)tartrate, the other by first resolving the side chain intermediate 2-(*N*-methyl-piperidyl-2′)-ethanol. Our observations of the number of products produced on oxidation by H_2O_2 required consideration of asymmetric centres other than the carbon of the piperidine ring. Asymmetric sulphoxides are chiral (Harrison et al 1926), thus, in the case of thioridazine-5-sulphoxide, four possible isomers may be obtained. Separation of the diastereoisomeric pairs from human plasma has recently been reported (Polkis et al 1982; Juenge et al 1983; Wells et al 1983). Similar arguments apply to thioridazine-2-sulphoxide and to thioridazine-*N*-oxide. If all three positions contain one oxygen, i.e. thioridazine-2,5,*N*-trioxide, there are 16 possible isomers.

MATERIALS AND METHODS

Reagents and reference materials

HPLC-grade methanol and acetonitrile were purchased from Fisons Scientific Apparatus, Loughborough, Leicestershire, UK. Other reagents were analytical grade, when available. Thioridazine, thioridazine-5-sulphoxide, thioridazine-2-sulphoxide, thioridazine-2-sulphone and *N*-desmethylthioridazine were gifts from Sandoz, Feltham, Middlesex, UK. (+)- and (−)-Thioridazine were provided by Dr Maurer, Sandoz, Basle, Switzerland. Diastereoisomeric 5-sulphoxides were obtained by H_2O_2 oxidation of the corresponding sulphide in acidic solution, by reaction with nitrous acid (Juenge et al 1983) or by leaving dilute methanolic solutions at room temperature (20 °C). Thioridazine-*N*-oxides were prepared by oxidizing with 3-chloroperbenzoic acid (Beckett et al 1974). The diastereoisomers were separated by liquid chromatography and examined by NMR.

High-performance liquid chromatography

Two systems were used. System I used an Altex Model 330 isocratic system with fixed wavelength (254 nm) detection. The stainless steel column, 250 × 4·5 mm i.d., was slurry packed with 5 μm silica particles (Spherisorb, Phase Separations, Queensferry, UK) in methanol. The eluent, which consisted of methanol–acetonitrile–0·88 NH_4OH–water, 500 : 500 : 0·25 : 3·75, by volume, was pumped at 2 ml min^{-1}. Samples were introduced via an Altex 210 injection valve fitted with a 20 μl sample loop. System II was a Laboratory Data Control Constametric III pump and a column of 3 μm silica gel (Spherisorb, Phase Separations), 150 × 4·6 mm i.d. The eluent was that described for System I or, for the preparative separation of the amine oxides, methanol.

Resolutions of enantiomers were attempted using either ionically bonded *N*-(3,5-dinitrobenzoyl)-D-phenylglycine or covalently bonded (*R*)-*N*-(α)-phenethyl-*N*′-triethoxysilyl-propylurea (Phase Separations). Hexane-isopropanol mixtures up to a maximum of 20% isopropanol were used with the ionic column and methanol with the covalent one.

Thin-layer chromatography

This was on plastic-backed silica gel plates, 0·2 mm thick (Whatman) containing fluorescent indicators.

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The plates were developed in chloroform–ethanol–0·88 NH_4OH, 80:10:1 by volume. The spots were located as shadows under short wave (250 nm) ultraviolet light. The groups present were characterized by fluorescence under long wave ultraviolet light (350 nm) and the colours developed after spraying with Folin and Ciocalteu's reagent or 50% H_2SO_4 (Table 1).

Nuclear magnetic resonance spectra

Spectra were obtained using dilute solutions in 100 atom % $CDCl_3$ on Brucker WH-80 or WH-400 spectrometers operated in the Fourier transform mode. Peaks were assigned on the basis of expected chemical shifts, by comparison with a series of model compounds and, for the *N*-methyl groups of the *N*-oxides, on the basis of W-coupling (Jackman & Sternhell 1969) between the protons of the axial methyl group and the two adjacent axial hydrogen atoms.

Biological material

Male, Wistar rats were housed in metabolic cages. Urine and faeces samples were collected for 24 h from control animals and from animals dosed with thioridazine hydrochloride (20 mg, i.p.). At the end of their time in cages (3–14 days) the rats were killed and hearts, livers, lungs and brains dissected for assay. Tissues were homogenized in isotonic KCl/phosphate buffer, pH 7·6 using a Potter-Elvejhem homogenizer, or, in the case of lung and heart, an Ultra-Turrax blender, to produce a 25% w/w homogenate.

In-vitro metabolic studies

These were carried out with 9000*g* supernatants from homogenized rat livers using standard techniques (Curry & Whelpton 1983). Aliquots were prepared for liquid chromatography by adding 3 volumes of methanol and centrifuging at 15 000 rev min^{-1} for 3 min. The supernatant was injected into the chromatograph without further treatment.

Solvent extraction and sample preparation

Plasma, urine or tissue homogenate (1 ml) was made alkaline with 2 M NaOH (1 ml) and extracted with heptane containing 1·5% v/v amyl alcohol (5 ml) or diethyl ether (5 ml). After centrifugation to separate the phases, the organic layer (4·0 ml) was transferred to a pointed tube and evaporated at 45 °C under a gentle stream of nitrogen. The residue was dissolved in methanol (100 µl) and samples injected into the chromatograph.

RESULTS AND DISCUSSION

The thin-layer chromatographic separations of diastereoisomeric 5-sulphoxide and *N*-oxides are given in Table 1. Thioridazine and its metabolites produce characteristic semiquinone free radical colours which are a useful guide to the type of ring substituents present (Table 1). Pairs of diastereoisomers produced the same free radical colours. Thioridazine *N*-oxides gave the same turquoise colour as thioridazine and were characterized by their reduction to thioridazine on treatment with acidic metabisulphite solution and NMR spectra. Other phenothiazine *N*-oxides undergo a similar reduction (Gaertner et al 1974; Curry & Evans 1976). Sulphoxides were reduced by zinc/HCl or by treatment with $TiCl_3$ solution. Sulphones were resistant to this treatment. The diastereoisomeric thioridazine-*N*-oxides were produced in unequal amounts (approximately 4 to 1), as would be expected. NMR analysis showed that the major isomer had an axial *N*-methyl group (at δ 2·77) attached to the piperidine ring, while the minor isomer has the *N*-methyl group (δ 2·91) in the equatorial position. Thus, the oxidizing agent prefers to approach the nitrogen lone pair of thioridazine (*N*-methyl at δ 2·29) equatorially, i.e. along the least hindered pathway.

By careful choice of eluent it was possible to resolve all the compounds of interest. Separation of the reference compounds provided by Sandoz is shown in Fig. 1A. Generally, the more lipophilic compounds eluted first. The long retention times of the desmethyl compounds probably reflect the greater basicity of the secondary amines. The 5-oxides racemized in dilute methanolic solutions. Fig. 1B includes diastereoisomeric sulphoxides obtained by leaving dilute methanolic solutions (10 µg ml^{-1}) of the

Table 1. R_F-values and semi-quinone free radical colours of reference materials.

No.	Compound	R_F	Colour	λ_{max} (nm)
1	TDZ-2-SO_2	0·52	salmon	510
2	TDZ	0·66	turquoise	640
3	TDZ-2-SO_2-5SO	0·37	—	—
3a		0·42	—	—
4	TDZ-5SO	0·44	turquoise*	640
4a		0·39	turquoise*	640
5	TDZ-2-SO	0·35	pink	525
6	TDZ-2,5-diSO	0·21	—	—
6a		—	—	—
7	NorTDZ	0·49	turquoise	640
8	NorTDZ-5-SO	0·27	turquoise*	640
8a		0·18	turquoise*	640
9	NorTDZ-2-SO	0·17	pink	525
10	TDZNO	0·30	turquoise	640
10a	TDZNO	0·34	turquoise	640

* After heating.

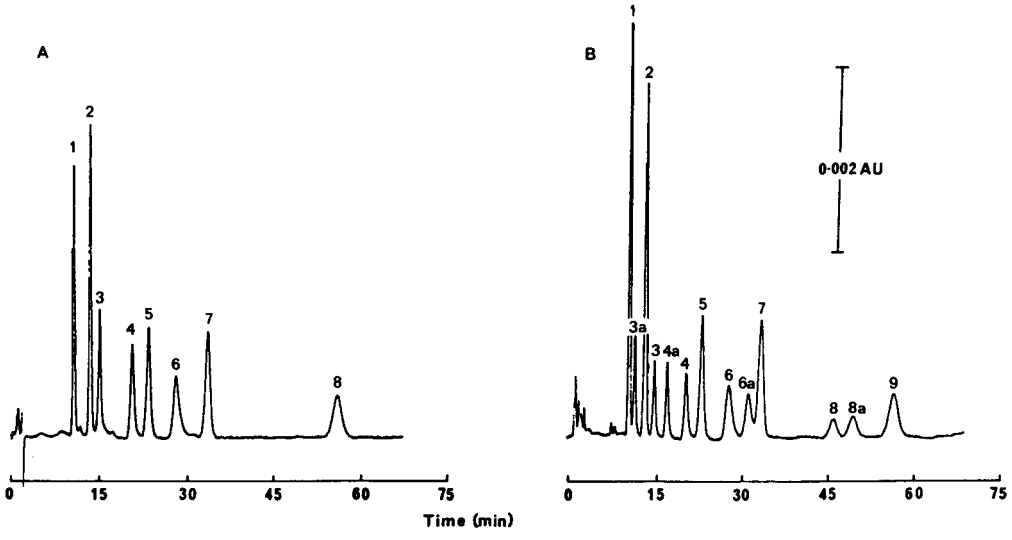

FIG. 1. (A) HPLC (System II) trace of reference compounds supplied by Sandoz Ltd. 1. Thioridazine-2-sulphone. 2. Thioridazine. 3. Thioridazine-2-sulphone-5-sulphoxide. 4. Thioridazine-5-sulphoxide. 5. Thioridazine-2-sulphoxide. 6. Thioridazine-2,5-disulphoxide. 7. Desmethylthioridazine. 9. Desmethylthioridazine-2-sulphoxide. (B) As (A), but including diastereoisomeric 5-oxides of compounds 3, 4, 6 and desmethylthioridazine-5-sulphoxides (8 and 8a).

reference 5-oxides at room temperature for eight days, or, for the desmethylthioridazine-5-oxides, by nitrous acid oxidation of desmethylthioridazine. The rate at which dilute solutions racemized was not affected by adding acid or alkali. Concentrated solutions (1 mg ml^{-1}) of the reference 5-oxides were stable for at least 12 months when stored at −20 °C.

Thioridazine-5-sulphoxide diastereoisomers were identified in lung and heart. Homogenates of lung contained a high proportion of sulphoxides, including the diastereoisomeric 5-sulphoxides (Fig. 2). The peaks corresponding to the 5-sulphoxide isomers were always of a similar size, which suggests there is no stereochemical selectivity when thioridazine is metabolized to its 5-sulphoxide. Muusze (1975) reported an unidentified metabolite which was present at very similar concentrations as thioridazine-5-sulphoxide in patients on long-term thioridazine therapy or following a single dose. This was probably the diastereoisomer thioridazine-5-sulphoxide. *N*-Desmethylthioridazine-5-sulphoxide diastereoisomers were identified in lung homogenates and were present in the same proportions (Fig. 2).

Traces of thioridazine-*N*-oxides were found in rat urine samples (Fig. 3). Their identification may affect the results of thioridazine assays. Amine oxides are thermally unstable and may be reduced to the parent compound and other products on GLC columns (Curry & Evans 1976). They are labile in alkaline solution and may revert to the parent compound to produce an overestimate of the concentration originally present (Whelpton 1978).

So far we have been unable to separate the diastereoisomers of thioridazine-2-sulphoxide.

Attempts to resolve enantiomers of thioridazine or its metabolites using the phenylglycine column were

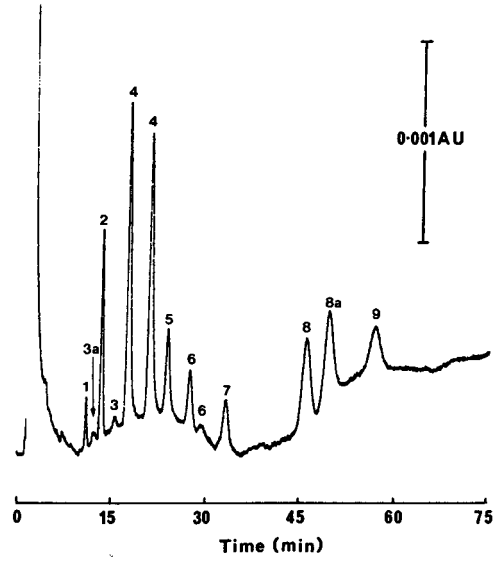

FIG. 2. HPLC trace of heptane-extractable material from homogenized rat lung after in-vivo treatment with thioridazine hydrochloride. Compounds as in Fig. 1.

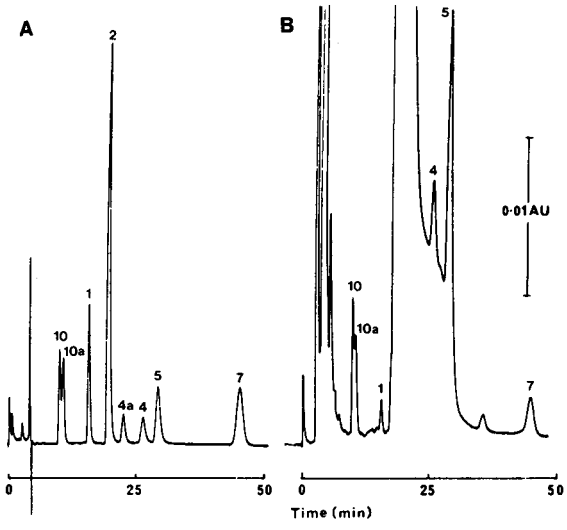

FIG. 3. (A) HPLC trace of selected reference compounds. Diastereoisomers of thioridazine *N*-oxide (10 and 10a), other compounds as Fig. 1. (B) HPLC trace of heptane-extractable material from rat urine.

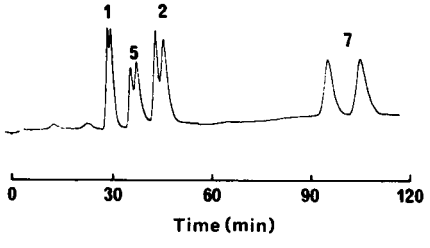

FIG. 4. Resolution of thioridazine (2) and some of its metabolites on the propylurea chiral column: thioridazine-2-sulphone (1), thioridazine-2-sulphoxide (5) and desmethyl thioridazine (7). Eluent: HPLC grade methanol.

unsuccessful, but partial resolutions were obtained with the propylurea column (Fig. 4). To date, pilot experiments have not shown any stereochemical preference in the metabolism of thioridazine. The residual thioridazine, after in-vitro incubation with liver homogenate was still racemic, as was the thioridazine extracted from rat urine.

That oxidation of thioridazine produces diastereoisomeric oxides precludes the danger that metabolites will not be identified, as in the past, or be incorrectly assigned. It would appear that earlier work on the metabolism of thioridazine may have underestimated the amounts of thioridazine-5-sulphoxide by 50%. The melting points of the isomers differ by 21 °C (Juenge et al 1983), and anyone working with a pure reference standard of thioridazine-5-sulphoxide with a sharp melting point will have one diastereoisomeric form (presumably consisting of a pair of enantiomers). Unless the material is characterized, e.g. by melting point, chromatographic properties, etc., it is not possible to be sure that workers in other laboratories are working with the same material.

Acknowledgements

We wish to thank the Nuffield Foundation for support under the Small Grants Scheme to purchase chiral HPLC columns, and Mr N. Mellor, Phase Separations Ltd., for providing the 'propylurea' chiral column. G. M. Watkins was supported by M.R.C. Grant No. 979/342/N.

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