Pharmaceutical Research, Vol. 4, No. 3, 1987

Research Article

Synthesis and Properties of Haptens for the Development of Radioimmunoassays for Thioridazine, Mesoridazine, and Sulforidazine

Bhaswat S. Chakraborty,[1] Manik S. Sardessai,[1] Theodore J. Jaworski,[1] Kamal K. Midha,[1] and Edward M. Hawes[1,2]

Received November 5, 1986; accepted February 23, 1987

For the separate development of radioimmunoassay procedures for thioridazine and its two major active metabolites, mesoridazine and sulforidazine, three haptens, respectively, 2-methylthio-, 2-methylsulfinyl-, and 2-methylsulfonyl-substituted 10-[2-[1-(2-carboxyethyl)-2-piperidinyl]ethyl]-10*H*-phenothiazine, were synthesized and characterized. Thioridazine hapten was coupled to bovine serum albumin, whereas the haptens for mesoridazine and sulforidazine were coupled to porcine thyroglobulin. The number of hapten residues per mole of carrier protein was determined in each case by an ultraviolet spectrophotometric method. Polyclonal antibodies to each hapten–protein conjugate were obtained in rabbits, and titers of the antisera were checked by evaluating their binding characteristics to the appropriate tritiated analyte. A hapten for the ring sulfoxide metabolite of thioridazine was also synthesized.

KEY WORDS: synthesis; properties; haptens; thioridazine; mesoridazine; sulforidazine.

INTRODUCTION

The piperidine type phenothiazine antipsychotic agent thioridazine (**1a**) (Fig. 1) is extensively biotransformed to numerous metabolites by various routes of metabolism, which include ring *S*-oxidation, side-chain *S*-oxidation, *N*-dealkylation, and aromatic hydroxylation (1,2). The side-chain sulfoxide and sulfone metabolites produced by *S*-oxidation of the 2-thioether group, known, respectively, as mesoridazine (**1b**) and sulforidazine (**1c**), are themselves marketed as antipsychotic agents with a reputedly greater potency than thioridazine. These two metabolites are generally considered to contribute to the clinical response in patients under chronic treatment with thioridazine. Thus, in studies attempting to correlate the clinical response of thioridazine with plasma concentrations, mesoridazine and sulforidazine should be measured (3,4).

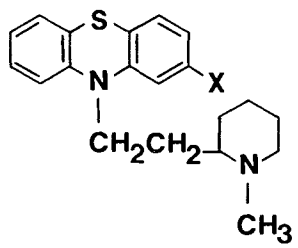
The chemical methods of analysis suitable for the quantitation of plasma concentrations of thioridazine, mesoridazine, and sulforidazine in patients under chronic treatment with thioridazine include gas–liquid chromatography (GC) with flame ionization (4,5) or nitrogen–phosphorus (6) detection and high-performance liquid chromatography (HPLC) with ultraviolet (UV) (7–9) or electrochemical detection (10). Regarding biological methods of analysis, radioreceptor assay has been used for the analysis of plasma samples from patients administered these drugs (11). Only in the case of thioridazine has a radioimmunoassay (RIA) method been reported (12). RIA methods, compared with chemical methods of analysis, generally require smaller biological samples and a much simpler preparation, if any, of the sample for analysis. Also, RIA methods are convenient, are readily applicable in routine analysis, and are able to handle large numbers of samples with a short turnaround time. In the case of the piperidine-type phenothiazine antipsychotic agents, the unbound plasma levels may be a better predictor of the clinical response than the total plasma levels (13). Since thioridazine, mesoridazine, and sulforidazine are extensively bound to plasma proteins, an ultrasensitive method of analysis, such as RIA, is required to measure their free plasma levels in clinical samples. The only report (14) on the measurement of free concentrations of thioridazine, mesoridazine, and sulforidazine in samples obtained from patients under chronic treatment with thioridazine utilized a GC method with electron capture detection and required the use of large serum samples and tedious sample preparations.

The published RIA procedure for the determination of thioridazine was developed by raising antibodies to a conjugate obtained from the hapten, *N*-(3-carboxypropionyl)desmethylthioridazine (12). However, many of the RIA procedures previously reported from these laboratories for phenothiazine antipsychotic agents and their major metabolites have been developed from antibodies raised to conjugates obtained from the *N*-(2-carboxyethyl)desmethyl analogue of the particular analyte (15–20). This paper describes the synthesis and characterization of the *N*-(2-carboxyethyl)des-

[1] College of Pharmacy, University of Saskatchewan, Saskatoon, Saskatchewan, Canada S7N 0W0.

[2] To whom correspondence should be addressed.

0724-8741/87/0600-0207$05.00/0



1 a, X=SCH_3
b, X=$SOCH_3$
c, X=SO_2CH_3

Fig. 1.

methyl analogues of thioridazine, mesoridazine, and sulforidazine, the use of protein conjugates of these haptens in the production of antibodies in rabbits, and the titers of the antisera. A hapten for the ring sulfoxide metabolite of thioridazine was also obtained as a result of the synthetic investigations.

MATERIALS AND METHODS

Thioridazine hydrochloride, mesoridazine besylate, and sulforidazine free base were gifts from Sandoz Pharmaceuticals, Dorval, Canada, and Sandoz Inc., East Hanover, N.J. The ring sulfoxide metabolites of thioridazine, mesoridazine, and sulforidazine were synthesized by modifying a literature procedure (21). The *N*-methyl tritiated derivatives of thioridazine, mesoridazine, and sulforidazine were custom-made through the Amersham Corporation, Oakville, Canada. Bovine serum albumin and porcine thyroglobulin were purchased from Sigma Chemical Co., St. Louis, Mo. Plastic sheets (E. Merck) precoated with silica gel, with or without a fluorescent indicator for thin-layer chromatography (TLC), and silica gel (60–200 mesh) for column chromatography were obtained from Terochem Laboratories Ltd., Edmonton, Canada, and J. T. Baker Chemical Company, Phillipsburg, N.J., respectively. All reagents except methyl acrylate were purchased from Aldrich Chemical Co., Milwaukee, Wis. Methyl acrylate was procured from Eastman Kodak Co., Rochester, N.Y. All solvents were reagent and/or HPLC grade and were used without further purification.

The removal of solvent from crude reaction mixtures was carried out under reduced pressure using a Buchi Rota-Vapor, Model RE120, Brinkmann Instruments, Rexdale, Canada. TLC spots were observed under shortwave UV light. All the melting points (m.p.) were determined in open glass capillaries with a Gallenkamp melting-point apparatus and are uncorrected. Proton magnetic resonance (^{1}H-NMR) spectra were determined on a Varian T-60 instrument in deuteriochloroform with tetramethylsilane as the internal reference. Low-resolution electron-impact mass spectra (MS) of probe samples were recorded on a VG Micromass MM16F instrument at 70 eV, equipped with a VG 2025 data system. Peaks $<m/z$ 75 or relative intensity <5% of the base peak were omitted, except for the base peak and diagnostic ion and molecular ion peaks. Microanalyses for samples dried over phosphorus pentoxide at 60°C under reduced pressure were performed at the Department of Chemistry, University of Saskatchewan or Guelph Laboratories, Guelph, Canada. Quantitative UV spectrophotometry was performed using a Pye Unicam SP 1700 instrument. Radiochemical purity of the *N*-methyl tritiated compounds was determined by TLC and HPLC.

For liquid scintillation counting, Ready Solve-MP was purchased from Beckman Instruments Inc. (Fullerton, Calif.). The dextran-coated charcoal suspension and various buffers were prepared in this laboratory. Liquid scintillation counting was performed in an LKB Rackbeta liquid scintillation counter equipped with an automatic quench compensation (Model 1215).

Synthesis of the Hapten for Thioridazine, 10-[2-[1-(2-Carboxyethyl)-2-piperidinyl]ethyl]-2-methylthio-10H-phenothiazine (4a). A mixture of 10-[2-(2-piperidinyl)ethyl]-2-methylthio-10*H*-phenothiazine (**2a**) (0.72 g, 2.0 mmol), synthesized in these laboratories (22) [m.p. of the hydrochloride salt, 208–211°C; lit. (23) m.p., 212–214°C], methyl acrylate (0.19 g, 2.2 mmol), and absolute methanol (30 ml) was maintained under gentle reflux for 24 hr. The solvent and excess methyl acrylate were removed under reduced pressure. The resulting oily residue, when chromatographed on a silica gel column with methanol in chloroform (1:10) as the elution solvent gave, after evaporation of the solvent, the ester **3a** (0.65 g, 73%) as a pale-yellow oil. ^{1}H NMR: δ1.12–1.94 (m,10, piperidine methylene groups, CH_2-piperidine), 2.42 (s,3, SCH_3), 2.70–3.14 (m,5, CH_2CH_2-ester, piperidine ring methine H), 3.64 (s,3, ester CH_3), 3.92 (t,2, CH_2-phenothiazine), and 6.72–7.24 ppm (m,7, ArH). MS: *m/z* 442($M^{+\cdot}$, 8), 356(7), 272(2), 271(13), 258(18), 245(32), 244(16), 226(8), 198(14), 197(18), 185(13), 184(16), 170(100), 112(8), 111(6), 98(64), and 84(32).

The ester (**3a**) (0.45 g, 1 mmol) was hydrolyzed by refluxing in methanol (15 ml) with NaOH (0.16 g, 4 mmol) and water (15 ml) for 2 hr. The methanol was removed under reduced pressure, and a further 15 ml of water was added. After washing with ether (3 × 10 ml), the aqueous phase was adjusted to pH 7.0 with 1 *M* HCl. The product was then extracted with methylene chloride (3 × 10 ml) and the dried ($MgSO_4$) extract was evaporated under reduced pressure to give the desired hapten (0.40 g, 93%) as white scales, m.p. 86–88°C. ^{1}H NMR: δ1.32–2.16 (m,10, piperidine methylene groups, CH_2-piperidine), 2.42(s,3, SCH_3), 2.72–3.24 (m,5, CH_2CH_2-acid, piperidine ring methine H), 3.92 (t,2, CH_2-phenothiazine), 6.72–7.24 (m,7, ArH), and 9.30 ppm (s,1, acid H which exchanges with D_2O). MS: *m/z* 428($M^{+\cdot}$, 2), 356(56), 272(2), 271(3), 258(22), 245(51), 244(18), 226(8), 198(6), 197(12), 185(12), 184(3), 156(4), 112(32), 111(28), 98(6), 84(100), and 72(36). A sample was purified for elemental analysis by column chromatography with silica gel as adsorbent and a mixture of 1-propanol and chloroform (1:1) as the eluting solvent.

Anal.—Calcd for $C_{23}H_{28}N_2O_2S_2$: C, 64.48; H, 6.58; N, 6.54. Found: C, 64.43; H, 6.54; N, 6.44.

Synthesis of the Hapten for Mesoridazine, 10-[2-[1-(2-Carboxyethyl)-2-piperidinyl]ethyl]-2-methylsulfinyl-10H-phenothiazine (4b). The intermediate ester **3b** was prepared from 10-[2-(2-piperidinyl)ethyl]-2-methylsulfinyl-10*H*-phenothiazine (**2b**) [synthesized in these laboratories (22); m.p. of the besylate salt, 146–148°C; lit. (24) m.p., 142–144°C] in the same manner as described for **3a** to yield 75% of a brown–yellow oil. ^{1}H NMR: δ1.23–2.64 (m,10, piperidine

methylene groups, CH_2-piperidine), 2.70 (s,3, SCH_3), 2.80–3.28 (m,5, CH_2CH_2-ester, piperidine methine H), 3.68 (s,3, ester CH_3), 4.00 (*t*,2, CH_2-phenothiazine), and 6.78–7.42 ppm (m,7, ArH). MS: *m/z* 458($M^{+\cdot}$, 11), 442(2), 372(6), 356(1), 287(1), 274(3), 271(1), 261(5), 260(2), 259(4), 258(4), 245(3), 244(1), 242(1), 198(5), 197(3), 185(3), 184(2), 170(100), 112(4), 111(3), 98(10), and 84(16).

The acid **4b** was obtained from **3b** by alkaline hydrolysis in the same manner as described for **4a** to yield 85% of pale yellow scales, m.p. 89–91°C. ^1H NMR: δ1.32–2.64 (m,10, piperidine methylene groups, CH_2-piperidine), 2.70 (s,3, SCH_3), 2.85–3.36 (m,5, CH_2CH_2-acid, piperidine methine H), 4.03 (t,2, CH_2-phenothiazine), 6.78–7.42 (m,7, ArH), and 8.73 ppm (s,1, acid H which exchanges with D_2O). MS: *m/z* 444($M^{+\cdot}$, 2), 428(3), 372(37), 356(6), 355(9), 288(1), 287(2), 274(8), 261(24), 260(8), 259(14), 258(21), 245(10), 244(10), 242(1), 198(5), 197(6), 185(4), 184(2), 156(26), 112(24), 111(15), 98(16), 84(100), and 72(11). A sample of **4b** for elemental analysis was prepared as for **4a**.

Anal. Calcd for $C_{23}H_{28}N_2O_3S_2$: C, 62.13; H, 6.35; N, 6.30. Found: C, 61.44; H, 6.51; N, 6.38.

Synthesis of the Hapten for Sulforidazine, 10-[2-[1-(2-Carboxyethyl)-2-piperidinyl]ethyl]-2-methylsulfonyl-10H-phenothiazine (4c). The intermediate ester **3c** was prepared from 10-[2-(2-piperidinyl)ethyl]-2-methylsulfonyl-10*H*-phenothiazine (**2c**) [synthesized in these laboratories (22); m.p., 146–148°C] in the same manner as described for **3a** to yield 66% of a bright yellow oil. ^1H NMR: δ1.06–3.00 (m,15, piperidine methylene groups, CH_2-piperidine, piperidine methine H, CH_2CH_2-ester), 3.06 (s,3, SCH_3), 3.66 (s,3, ester CH_3), 4.05 (t,2, CH_2-phenothiazine), and 6.78–7.65 ppm (m,7, ArH). MS: *m/z* 474($M^{+\cdot}$, 8), 388(7), 304(1), 303(2), 290(5), 277(6), 276(2), 258(2), 198(4), 197(4), 185(1), 184(1), 170(100), 112(6), 111(4), 98(39), and 84(26).

The acid **4c** was prepared from **3c** by alkaline hydrolysis in the same manner as described for **4a** to give 92% of yellow scales, m.p. 100–102°C. ^1H NMR: δ1.40–3.00 (m,15, piperidine methylene groups, CH_2-piperidine, piperidine methine H, CH_2CH_2-acid), 3.06 (s,3, SCH_3), 4.05 (t,2, CH_2-phenothiazine), 6.78–7.65 (m,7, ArH), and 10.64 ppm (s,1, acid H which exchanges with D_2O). MS: *m/z* 460($M^{+\cdot}$, 7), 388(74), 304(2), 303(5), 290(25), 277(40), 276(8), 258(7), 198(9), 197(12), 185(2), 184(2), 156(15), 112(43), 111(33), 98(15), 84(100), and 72(25). A sample of **4c** for elemental analysis was prepared as for **4a**.

Anal. Calcd for $C_{23}H_{28}N_2O_4S_2$: C, 59.97; H, 6.13; N, 6.08. Found: C, 59.71, H, 5.73; N, 6.17.

Synthesis of the Hapten for the Ring Sulfoxide Metabolite of Thioridazine, 10-[2-[1-(2-Carboxyethyl)-2-piperidinyl]ethyl]-2-methylthio-10H-phenothiazine-5-sulfoxide (5). A solution of **4a** (0.43 g, 1 mmol) in 2 *M* HCl (30 ml) was added dropwise to a sodium nitrite solution (10%) with continuous stirring over a period of 30 min, after which the stirring was continued for another 1 hr. The mixture was extracted with methylene chloride (3 × 10 ml), and the organic solvent removed under reduced pressure. The deep-orange glassy residue was dissolved in water, and the solution adjusted to pH 7.0 with 1 *M* NaOH and saturated with solid NaCl. The product was then extracted with methylene chloride (3 × 10 ml), and the dried ($MgSO_4$) extract was evaporated under reduced pressure to give **5** (0.38 g, 86%) as yellow scales, m.p. 96–100°C. ^1H NMR: δ1.26–2.50 (m,10, piperidine methylene groups, CH_2-piperidine), 2.58 (s,3, SCH_3), 2.60–3.32 (m,5, CH_2CH_2-acid, piperidine ring methine H), 4.35 (t,2, CH_2-phenothiazine), 6.92–8.00 (m,7, ArH), and 9.40 (s,1, acid H which exchanges with D_2O). MS: *m/z* 444($M^{+\cdot}$, 1), 428(1), 427(1), 372(3), 356(14), 355(16), 288(1), 287(1), 274(1), 261(1), 260(6), 259(16), 258(42), 245(15), 244(11), 243(2), 242(1), 226(14), 198(5), 197(3), 185(9), 184(2), 156(1), 112(6), 111(7), 98(3), 84(100), and 72(11). A sample of **5** for elemental analysis was prepared as for **4a**.

Anal. Calcd for $C_{23}H_{28}N_2O_3S_2$: C, 62.13; H, 6.35; N, 6.30. Found: C, 61.94; H, 6.00; N, 6.18.

Coupling of 4a to Bovine Serum Albumin. Compound **4a** was coupled to bovine serum albumin by a modified mixed anhydride method (15,25,26). Compound **4a** (0.22 g, 0.51 mmol) and isobutyl chloroformate (67 μl, 0.51 mmol) were dissolved in freshly distilled dioxane (4 ml), and the solution was cooled to 15°C and allowed to stand for 30 min. The mixture was then slowly added in 0.5-ml aliquots to a stirred, cooled (4°C) solution of bovine serum albumin (0.58 g, 0.0083 mmol) in a mixture of water (25 ml) and dioxane (8 ml), which had been adjusted to pH 9.3 with 2 *M* NaOH. Throughout the addition, the pH was maintained at pH 9.3 with 2 *M* NaOH. After the addition was complete, the clear solution was allowed to stand at 4°C for 12 hr with continuous stirring.

The solution was dialyzed at room temperature, initially every 15 min against bicarbonate buffer (0.2 *M*, pH 8.0, 3 × 1000 ml) and acetate buffer (0.2 *M*, pH 5.0, 3 × 1000 ml) and then, finally, against distilled water continuously for 4 hr. After lyophilization, the conjugate **6a** was obtained as a white crystalline solid (0.72 g). A blank was prepared in the same manner but without **4a**. The number of hapten residues coupled per mole of bovine serum albumin was calculated by the UV method (25,26) to be 31 (**6a**; $n = 31$).

The procedure was repeated twice, decreasing by half the relative amount of bovine serum albumin (0.29 g, 0.0041 mmol) with **4a** (0.22 g, 0.51 mmol). These conjugates (0.45 g, 0.48 g) were determined to have 27 and 31 hapten residues per mole of bovine serum albumin (**6a**; $n = 27, 31$).

Coupling of 4b to Porcine Thyroglobulin. Compound **4b** was coupled to porcine thyroglobulin by a modified carbodiimide method (27,28). A solution of porcine thyroglobulin (0.36 g, 0.00053 mmol) in water (20 ml) was added to a solution of **4b** (0.18 g, 0.4 mmol) in water (20 ml). To this mixture, a solution of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (1.23 g, 6.4 mmol) in 10 ml of water was added dropwise with stirring, maintaining the pH between 7.0 and 7.6 by the addition of 0.1 *M* NaOH. The solution was allowed to stand for 4 hr at room temperature with continuous stirring. The solution was first exhaustively dialyzed against phosphate buffer (0.1 *M*, pH 7.6, 5 × 1000 ml) containing 0.1% ascorbic acid and then, finally, against distilled water. After lyophilization, the conjugate was obtained as a pale-yellow crystalline solid (0.42 g). A blank was prepared by subjecting thyroglobulin to the same coupling conditions but without the hapten. The number of hapten residues per mole of thyroglobulin was calculated by the UV method (25,26) to be 114 (**6b**; $n = 114$).

Coupling of 4c to Porcine Thyroglobulin. Compound **4c** was coupled to porcine thyroglobulin by a modified carbodiimide method (27,28) in the same manner as described for

the coupling of **4b**. Compound **4c** (0.19 g, 0.4 mmol) and porcine thyroglobulin (0.36 g, 0.00053 mmol) gave 0.54 g of the conjugate **6c** as yellow crystals, after lyophilization. The number of hapten residues per mole of porcine thyroglobulin was calculated by the UV method (25,26) to be 64 (**6c**; n = 64).

Immunization. Each hapten–protein conjugate (4 mg) was emulsified with sterile normal saline (2.0 ml) and Freund's complete adjuvant (GIBCO, Grand Island, N.Y.) (2.0 ml). Each conjugate was administered to at least three New Zealand white female rabbits (Animal Resources Centre, University of Saskatchewan, Saskatoon, Canada) by the injection of 0.5 ml of the emulsion into each shoulder. Thereafter, at 2-week intervals, the injections were repeated with an emulsion prepared as described above, except that Freund's complete adjuvant was replaced by Freund's incomplete adjuvant. The antiserum titer of each rabbit was checked by evaluating the binding characteristics with the appropriate tracer 1 week after the fifth injection, when the route of administration was changed to intravenous. The intravenous injections containing the same dose in 0.25 ml normal saline were administered through the marginal vein of the rabbit. In the experience of the authors (15–20), the selectivity of the antiserum does not change after five immunizations, whereas intravenous boosters at this stage lead to improved sensitivity with maintained selectivity.

The intravenous doses were repeated every 2 weeks, and the titers of the antisera checked 1 week after each administration. The rabbits which produced antisera of adequate titer (at least 1 in 250) were sacrificed and each antiserum was lyophilized and stored at −20°C.

Radioimmunoassay Procedures. All experimental manipulations were carried out under subdued light. A working antiserum solution was prepared by dissolving the appropriate lyophilized antiserum in phosphate buffer (0.2 *M*, pH 7.2) at the dilution indicated in Table I. Two hundred microliters of this solution was used in each assay tube.

To each 12 × 75-mm polystyrene tube containing a 200-μl plasma sample (standard or from a dosed volunteer) was added 200 μl of phosphate buffer (0.2 *M*, pH 7.2), and 100 μl of the appropriate working tracer solution (14,000–18,000 cpm). The contents of the tubes were mixed (Vortex) for 10 sec, and the appropriate working antiserum solution was added. All the additions were carried out in an ice bath. The contents of each tube were mixed well for 10 sec and incubated under the experimentally determined optimum conditions for the assay (e.g., at 4°C for 2 hr in the case of the thioridazine RIA procedure). To each of the tubes 1 ml of cold dextran-coated charcoal suspension was added, then mixed well, and the tubes were incubated for a suitable time (e.g., for 20 min in the case of the thioridazine RIA procedure) at 4°C. The mixture was then centrifuged (1720*g*, 10 min, 4°C) and the supernatant was decanted into a scintillation vial containing 10 ml of scintillation cocktail, mixed well, and counted for 5 min.

Table I. Rabbit Antiserum Titers of Hapten–Protein Conjugates for Thioridazine, Mesoridazine, and Sulforidazine[a]

Immunizing antigen[b]	Number of positives/number tested	Number of intravenous boosters	Titer[c]
6a, n = 31	1/3	4	1:600 (1)
6a, n = 27	3/4	2	1:3,000 (1)
			1:3,500 (2)
6a, n = 31	1/3	3	1:3,000 (1)
6b, n = 114	4/4	3	1:2,000 (1)
			1:4,000 (1)
			1:5,500 (1)
			1:7,000 (1)
6c, n = 64	4/4	1	1:8,000 (1)
			1:9,000 (1)
			1:10,000 (2)

[a] Titer values of 1:250 or less are not recognized.
[b] n = the number of hapten residues per mole of protein.
[c] The number of rabbits giving this titer in parentheses.

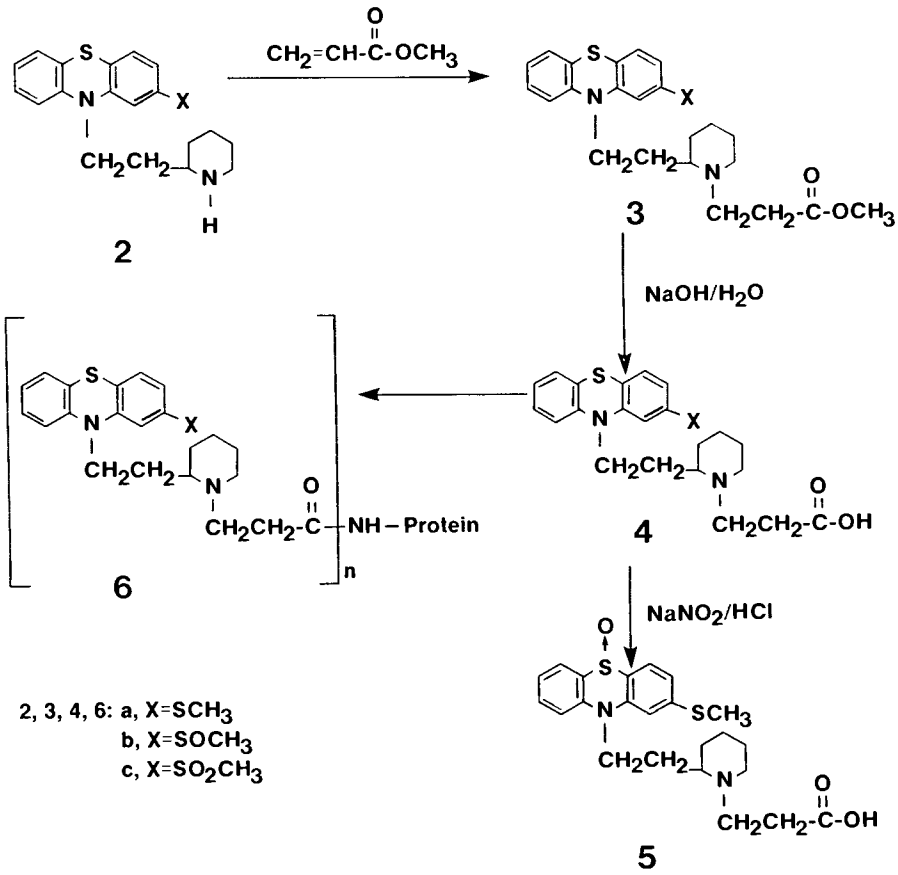
RESULTS AND DISCUSSION

In order to obtain haptens where a carboxylic acid group could be coupled to a suitable protein, the structures of thioridazine, mesoridazine, and sulforidazine were modified such that in each case, the *N*-methyl group was replaced by a *N*-(2-carboxyethyl) group. The synthetic route to these three haptens involved treatment of the appropriate *N*-desmethyl compound with methyl acrylate and subsequent treatment of the resultant ester with base (Scheme I). Since a suitable recrystallization solvent could not be found for each hapten, samples for elemental analysis were obtained by purification involving column chromatography. All three haptens and their methyl esters gave ^{1}H NMR and MS entirely consistent with the assigned structures.

In each case, the MS showed a molecular ion and fragmentations which could be rationalized in terms of the structures of the compounds. Some of the major diagnostic ions of the MS of all six compounds (**3a–c**, **4a–c**) are shown in Scheme II, with the origins of the ions indicated in parentheses. As reported (17,29) for other β-aminocarboxylic acids, **4a**, **4b**, and **4c** lose a neutral molecule of acrylic acid with a transfer of hydrogen, giving prominent ions at *m/z* 356, 372, and 388, respectively. Also, when the charge resides on the acrylic acid molecule, the ion at *m/z* 72 is formed. A similar loss of a neutral methyl acrylate molecule from the corresponding methyl esters (**3a**, **3b**, **3c**) results in the ions at *m/z* 356, 372, and 388, respectively. The presence of ions at *m/z* 244 (**3a**, **4a**), 260 (**3b**, **4b**), and 276 (**3c**, **4c**) is due to the cleavage of the *N*–C bond between the phenothiazine ring system and the *N*-10 side chain, with the charge being accommodated on the phenothiazine ring system (15). A similar cleavage with a transfer of hydrogen gives rise to the ions at *m/z* 245 (**3a**, **4a**), 261 (**3b**, **4b**), and 277 (**3c**, **4c**). The loss of $^{\cdot}CH_2CH(CH_2)_4NCH_2CH_2COOCH_3$ from the esters or $^{\cdot}CH_2CH(CH_2)_4NCH_2CH_2COOH$ from the acids leads to the formation of diagnostic ions at *m/z* 258, 274, and 290, respectively, arising from the pairs **3a** and **4a**, **3b** and **4b**, and **3c** and **4c**.

The cleavage of the C–C bond between the *N*-10 ethyl side chain and the piperidine ring gives rise to abundant piperidinium ions in the mass spectra of all six compounds. In fact in the case of the esters (**3a**, **3b**, **3c**) the base peak at *m/z* 170 arises from such a fragmentation; however, in the case of the acids (**4a**, **4b**, **4c**), likely due to the additional loss of a neutral acrylic acid moiety, the base peak appears at *m/z* 84.

Regarding side-chain sulfoxide compounds (**3b** and **4b**),



Scheme I

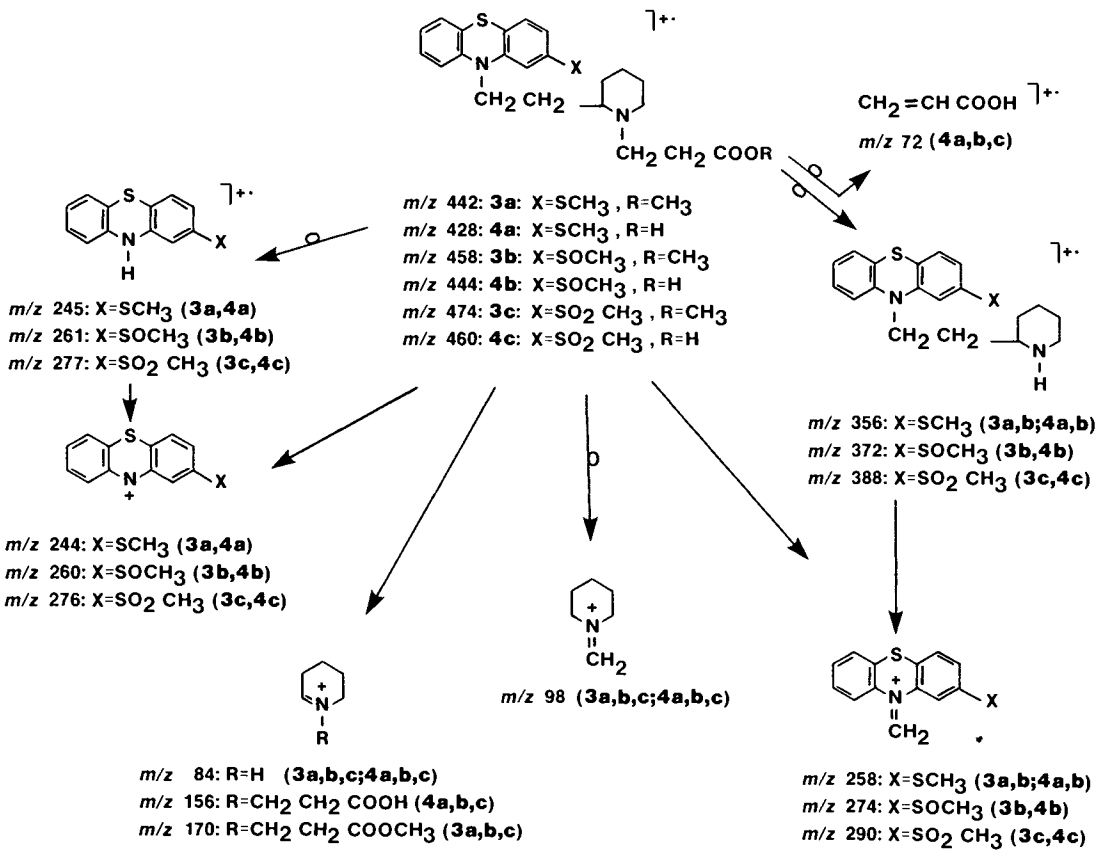
associated with the ions retaining the phenothiazine ring was in each instance a further ion which could be explained in terms of the facile loss of the sulfoxide oxygen atom, e.g., *m/z* 372 and 356 and *m/z* 274 and 258.

In early synthetic work, an alternative route to the hapten for mesoridazine (**4b**) was investigated, which involved oxidation of the hapten for thioridazine (**4a**). Selective oxidation of this molecule is difficult, as readily oxidizable centres include the ring sulfur, side-chain sulfur, and piperidine nitrogen. Indeed, oxidation of the hapten for thioridazine with hydrogen peroxide as oxidant gave a complex mixture of products, which proved difficult to separate by column chromatography. On the other hand, oxidation with sodium metaperiodate was successful in that the high-resolution ^{1}H NMR spectrum of the crude reaction mixture indicated that the major product was the hapten for mesoridazine. However, purification by column chromatography could not be accomplished due to the coelution of a minor component of the reaction mixture. The high-resolution ^{1}H NMR spectrum of the crude reaction mixture indicated that this minor component was likely a hapten for the ring sulfoxide metabolite of thioridazine (**5**). Since nitrous acid has been successfully used as a reagent to oxidize thioridazine selectively to its ring sulfoxide metabolite (21), the hapten for thioridazine was also treated with the reagent. In fact, the hapten for the ring sulfoxide metabolite of thioridazine was isolated in a high yield (86%) and subsequently characterized (elemental analysis, ^{1}H NMR, MS).

A conjugate of this hapten was not prepared at this stage. Despite the reported inactivity of the ring sulfoxide metabolite of thioridazine in pharmacological screens for antipsychotic activity (30), the need to raise antibodies to this metabolite could arise in future studies, because some workers have reported that thioridazine ring sulfoxide is present in the plasma of schizophrenic patients at higher levels in poor responders than good responders to thioridazine (31).

The haptens for thioridazine, mesoridazine, and sulforidazine were successfully coupled to protein. In the case of thioridazine, a modified mixed anhydride method (15,25,26) was used to couple the hapten to bovine serum albumin. The haptens for mesoridazine and sulforidazine were coupled to thyroglobulin by a modified carbodiimide method (27,28). The chemical reactivity of sulfoxide functional groups with anhydride reagents precludes the use of the mixed anhydride method as the coupling procedure for the hapten for mesoridazine.

In the initial coupling of the hapten for thioridazine to bovine serum albumin, 31 hapten residues were coupled to each mole of bovine serum albumin. In order to investigate whether the number of hapten residues coupled to 1 mol of the protein influences the quality of the thioridazine antiserum in terms of specificity and sensitivity, two further coupling experiments were performed where, in comparison to the initial experiment, the ratio of the amount of hapten to the amount of bovine serum albumin was increased twofold. However, for both the latter coupling experiments, the number of hapten residues found to be coupled per mole of



Scheme II

bovine serum albumin (n = 27, 31) was similar to that in the first experiment (n = 31). Porcine thyroglobulin, which was used as a carrier protein to couple the other two haptens, has theoretically almost 10 times as many acid binding sites as bovine serum albumin. In fact, the conjugates for mesoridazine and sulforidazine were obtained, respectively, with 114 and 64 of the appropriate hapten residues coupled per mole of thyroglobulin.

Separate groups of three or four New Zealand white female rabbits were immunized with each hapten–protein conjugate, as described under Materials and Methods. The preliminary asessment of the antisera so produced, together with the number of intravenous booster shots required, is presented in Table I. The immunization of each group of rabbits produced at least one polyclonal antiserum with a titer value of greater than 1:250. Both bovine serum albumin and thyroglobulin were successfully used as carrier proteins in that for each of these proteins, at least one antiserum was produced with a titer value of at least 1:3000. All those antisera with titers of at least 1:600 for a particular analyte were examined as to their cross-reactivity according to Abraham's criteria (32) with the other two analytes and the sulfoxide metabolites of thioridazine, mesoridazine, and sulforidazine. On the basis of these data the antiserum of each analyte most suitable for RIA development was subsequently chosen. Each selected antiserum showed minimal cross-reaction with the other two analytes and the actual determined values were as follows: thioridazine antiserum (1:3000 titer; immunizing antigen 6a; n = 31), 4% mesoridazine and sulforidazine; mesoridazine antiserum (1:7000 titer), 1% thioridazine and 4% sulforidazine; and sulforidazine antiserum (1:10,000 titer), <1% thioridazine and 2% mesoridazine. Also, each of the three antisera showed no significant (<1%) cross-reaction with any of the three sulfoxide metabolites. The general RIA procedure developed from the selected antisera is described in Materials and Methods.

The further characterization of these three antisera and the details of each RIA procedure for thioridazine (33), mesoridazine (34), and sulforidazine (35), which are capable in each case of quantitating the analyte, respectively, down to at least 0.4, 0.2, and 0.2 ng/ml using a 200-μl plasma sample, are separately published elsewhere. These RIA procedures were compared with HPLC methods by determining the concentrations of the respective analyte in plasma samples from five human volunteers over 72 hr after the administration of a single 50-mg oral dose of thioridazine hydrochloride (33–35). In each case there was a good correlation between the assay values (n = 55) determined by RIA as compared with HPLC ($r^2 > 0.91$). Furthermore, each of the three present RIA procedures had sufficient sensitivity to determine analyte concentrations in all plasma samples (n = 55) obtained up to 72 hr. The suitability of these procedures for use in relative bioavailability studies and for monitoring plasma concentrations in patients, including the free (protein unbound) levels, is being evaluated.

ACKNOWLEDGMENTS

Medical Research Council of Canada Operating Grants MT-7838 and PG-34 are gratefully acknowledged. We are thankful to Dr. G. McKay and Mr. R. W. Edom for running the mass spectra and the Canadian Red Cross for the generous gifts of plasma.

REFERENCES

1. K. Zehnder, F. Kalberer, W. Kreis, and J. Rutschmann. *J. Biochem. Pharmacol.* **11**:535–550 (1962).
2. A. S. Papadopoulos, J. L. Crammer, and D. A. Cowan. *Xenotiotica* **15**:309–316 (1985).
3. R. Axelsson and E. Martensson. *Curr. Ther. Res.* **24**:232–242 (1978).
4. A. S. Papadopoulos, T. G. Chand, J. L. Crammer, and S. Lader. *Br. J. Psychiat.* **136**:591–596 (1980).
5. E. Martensson, G. Nyberg, and R. Axelsson. *Curr. Ther. Res.* **18**:687–700 (1975).
6. A. Shvartsburd, V. Nwokeafor, and R. C. Smith. *Psychopharmacology (Berlin)* **82**:55–61 (1984).
7. T. Skinner, R. Gochnauer, and M. Linnoila. *Acta Pharmacol. Toxicol.* **48**:223–226 (1981).
8. C. D. Kilts, K. S. Patrick, G. R. Breese, and R. B. Mailman. *J. Chromatogr.* **231**:377–391 (1982).
9. G. McKay, J. K. Cooper, T. Gurnsey, and K. K. Midha. *LC Mag.* **3**:256–258 (1985).
10. A. L. Stoll, R. J. Baldessarini, B. M. Cohen, and S. P. Finklestein. *J. Chromatogr.* **307**:457–463 (1984).
11. S. H. Snyder. *Am. J. Psychiat.* **138**:460–464 (1981).
12. J. Rosenthaler, W. L. Pacha, S. Koeberle, and T. Beveridge. In E. Usdin, H. Eckert, and I. S. Forrest (eds.), *Phenothiazines and Structurally Related Drugs: Basic and Clinical Studies*, Elsevier/North-Holland, New York, 1980, pp. 121–124.
13. E. C. Dinovo, H. Pollack, and L. A. Gottschalk. *Methods Find. Exp. Clin. Pharmacol.* **6**:143–146 (1984).
14. G. Nyberg, R. Axelsson, and E. Martensson. *Eur. J. Clin. Pharmacol.* **14**:341–350 (1978).
15. J. W. Hubbard, K. K. Midha, I. J. McGilveray, and J. Cooper. *J. Pharm. Sci.* **67**:1563–1571 (1978).
16. P. K. F. Yeung, J. W. Hubbard, J. K. Cooper, and K. K. Midha. *J. Pharmacol. Exp. Ther.* **226**:833–838 (1983).
17. E. M. Hawes, H. U. Shetty, J. K. Cooper, G. Rauw, G. McKay, and K. K. Midha. *J. Pharm. Sci.* **73**:247–250 (1984).
18. M. Aravagiri, E. M. Hawes, and K. K. Midha. *J. Pharm. Sci.* **73**:1383–1387 (1984).
19. P. K. F. Yeung, G. McKay, I. A. Ramshaw, J. W. Hubbard, and K. K. Midha. *J. Pharmacol. Exp. Ther.* **233**:816–822 (1985).
20. M. Aravagiri, E. M. Hawes, and K. K. Midha. *J. Pharm. Sci.* **74**:1196–1202 (1985).
21. E. C. Juenge, C. E. Wells, D. E. Green, I. S. Forrest, and J. N. Shoolery. *J. Pharm. Sci.* **72**:617–621 (1983).
22. B. S. Chakraborty, Unpublished results.
23. Neth. Patent 6 516 316 (1966). *Chem. Abstr.* **65**:15393 (1966).
24. Neth. Patent 6 600 184 (1966). *Chem. Abstr.* **66**:2580r. (1967).
25. B. F. Erlanger, F. Borek, S. M. Beiser, and S. Lieberman. *J. Biol. Chem.* **228**:713–727 (1957).
26. B. F. Erlanger, F. Borek, S. M. Beiser, and S. Lieberman. *J. Biol. Chem.* **234**:1090–1094 (1959).
27. J. C. Sheehan and J. J. Hlavka. *J. Org. Chem.* **21**:439–441 (1956).
28. J. W. Hubbard, K. K. Midha, J. K. Cooper, and C. Charette. *J. Pharm. Sci.* **67**:1571–1578 (1978).
29. J. W. Hubbard, K. K. Midha, J. K. Cooper, and C. Charette. *Can. J. Pharm. Sci.* **15**:89–93, (1980).
30. C. D. Kilts, D. L. Knight, R. B. Mailman, E. Widerlov, and G. R. Breese. *J. Pharmacol. Exp. Ther.* **231**:334–342 (1984).
31. G. Sakalis, L. J. Traficante, and S. Gershon. *Curr. Ther. Res.* **21**:720–724 (1977).
32. G. E. Abraham. *J. Clin. Endocrinol. Metab.* **29**:866–870 (1969).
33. B. S. Chakraborty, E. M. Hawes, J. Rosenthaler, and K. K. Midha. *Ther. Drug Monit.* (in press).
34. B. S. Chakraborty, E. M. Hawes, and K. K. Midha. *Ther. Drug Monit.* (in press).
35. B. S. Chakraborty, E. M. Hawes, and K. K. Midha. *Ther. Drug Monit.* (submitted for publication).