METABOLISM OF SULFINPYRAZONE SULFIDE AND SULFINPYRAZONE BY HUMAN LIVER MICROSONES AND cDNA-EXPRESSED CYTOCHROME P450s

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ABSTRACT:

Human liver microsomes catalyze the oxidation of sulfinpyrazone sulfide (SPZS) to a variable mixture of sulfinpyrazone (SPZ) enantiomers and two minor phenolic metabolites. In one, the thiophenyl ring is hydroxylated, whereas in the second an N-phenyl ring is hydroxylated. SPZ is further oxidized to sulfinpyrazone sulfone (SPZO) and a minor polar metabolite that also has an N-phenyl ring hydroxylated. Determination of the metabolism of SPZ and SPZS under modified incubation conditions of prior heat treatment, higher pH, and the presence of detergent indicated that the formation of SPZ was cytochrome P450 (P450)- but not flavin monooxygenase-dependent. Specific P450 inhibitors (sulfaphenazole, quinidine sulfate, coumarin, diethyldithiocarbamic acid, trelandomycin, and furafylline) and specific cDNA-expressed P450s were used to identify the major isoforms responsible for the oxidation of SPZS to SPZ and SPZ to SPZO. Both P450 2C9 and P450 3A4 were responsible for the oxidation of SPZS to SPZ, whereas P450 3A4 alone catalyzed the further oxidation of SPZ and SPZO. SPZS was found to be metabolized by P450 2C9 to SPZ with a high degree of enantiomeric selectivity (9:1) and a $K_m$ comparable with its previously determined $K_m$ for inhibition of the P450 2C9-dependent 7-hydroxylation of (S)-warfarin (WARF). In contrast, the P450 3A4-catalyzed oxidation of SPZS to SPZ proceeded with the same enantioselectivity but to a much lesser degree (58:42). These results provide evidence that the metabolism of both (S)-WARF and SPZS is mediated by a common enzyme, P450 2C9, which is central to understanding the WARF-SPZ interaction and SPZS-mediated drug interactions in general.

SPZ² (Fig. 1) is a sulfoxide-containing uricosuric agent. In common with other sulfoxide-containing drugs, it is susceptible to both oxidative and reductive metabolism. At least nine metabolites have been identified in animals and humans (Dieterle et al., 1975, 1980; Dieterle and Faigle, 1981; Kirstein Pedersen and Jakobsen, 1981a). Structurally, all the metabolites except one consist of compounds with the sulfur atom in various states of oxidation (sulfide, sulfoxide, sulfone), either alone or in combination with a para hydroxy N-phenyl group or a C(4)-glucuronide. The lone exception is a SPZ metabolite that has the 4-position hydroxylated. During multiple dose treatment in humans, the sulfide (SPZS) and sulfone (SPZO) metabolites (Fig. 1) appeared as the major plasma metabolites (Kirstein Pedersen and Jakobsen, 1981b). At steady state, the plasma concentration of SPZS was found to be similar to or higher than that of the parent drug, SPZ (Rosenkranz et al., 1983). The possibility that in plasma SPZS can accumulate to levels in excess of SPZ is clinically significant because, relative to SPZ, it is a more potent inhibitor of both P450 2C9 (He et al., 1995) and platelet aggregation (Kirstein Pedersen and Fitzgerald, 1985).

SPZ has been implicated as an important factor in the genesis of metabolically based inhibitory drug interactions when coadministered with other drugs, such as WARF (Bailey and Reddy, 1980; Miners et al., 1982a), phenytoin (Hansen et al., 1980), and tolbutamide (Miners et al., 1982b). More recent work on the WARF-SPZ interaction suggested that the molecular basis for the interaction is the inhibition of the P450 2C9-catalyzed oxidation of (S)-WARF to its inactive (S)-6- and (S)-7-hydroxyWARF metabolites (Toon et al., 1986; Rettie et al., 1989, 1992). Because the inhibitory effect of SPZS on P450 2C9 in vitro was more than 10-fold that of SPZ (He et al., 1995) and because it attained plasma concentrations at least equal to those of SPZ during multiple dosing of SPZ (Rosenkranz et al., 1983) we concluded that in vivo SPZS is the major inhibitor of P450 2C9 and the primary cause of the clinical interaction (He et al., 1995).

Although it was clear that SPZS is formed by reductive metabolism mediated by gut bacteria (Strong et al., 1984), the reverse reaction, oxidation back to SPZ, had not been characterized in humans, although it is known to occur in rats (Renwick et al., 1982) and rabbits (Kuo and Ritschel, 1986). In view of the apparent importance of SPZS in mediating the WARF-SPZ drug interaction, the major goals of this investigation were to identify the enzyme(s) responsible for the formation of any oxidative metabolites, in vitro, and to establish their structures. In particular, we wished to characterize the re-oxidation of SPZS back to SPZ.

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2 Abbreviations used are: SPZ, sulfinpyrazone; CUM, coumarin; DDC, diethyldithiocarbamic acid; FUR, furafylline; FMO, flavin monooxygenase; P450, cytochrome P450; QUI, quinidine sulfate; RT, retention time; SFZ, sulfaphenazole; SPZO, sulfinpyrazone sulfone; SPZS, sulfinpyrazone sulfide; TAO, trelandomycin; WARF, warfarin; HPLC, high-performance liquid chromatography; MS/MS, mass spectrometry/mass spectrometry.
Fig. 1. Structures of SPZ and its two major metabolites, SPZS and SPZO.

Fig. 2. HPLC analysis of metabolites formed from incubation of liver microsomes from donor HL120 with SPZS where the peak at Rt 11.7 is the internal standard, (R)-(+)methyl-p-tolylsulfoxide, the peak at Rt 14.3 is metabolite A, the peak at Rt 15.8 is metabolite B, the peak at Rt 21.3 is SPZ, and the peak at Rt 42.8 is SPZS (a) and SPZ. where the peak at Rt 6.8 is metabolite C, the peak at Rt 11.8 is the internal standard, (R)-(+)methyl-p-tolylsulfoxide, the peak at Rt 18.6 is SPZO, and the peak at Rt 21.1 is SPZ (b).
Materials and Methods

Chemicals. SPZ and SPZS were gifts from Ciba Geigy Co. (New York, NY). SFZ, QUI, CUM, DDC, and TAO were obtained from Sigma Chemical Co. (St. Louis, MO). FUR was a gift from Dr. K. Kunze (Kunze and Trager, 1993). Lubrol was purchased from ICN Biochemicals (Cleveland, OH). (R)-Methyl-p-tolylsulfoxide was purchased from Aldrich Chemical Co. (Milwaukee, WI). Methanol and dichloromethane (Optima HPLC grade) were purchased from Fisher Scientific Co. (Pittsburgh, PA).

Synthetic Procedures. SPZS. Finely powdered, hydrogen-reduced iron (2.8 g, 0.05 mol) was washed with 10 ml of 5% HCl with vigorous shaking; the acid was decanted and the powder rinsed twice with glacial acetic acid. A suspension of the washed iron powder in 16.5 ml of glacial acetic acid was added to a 50-ml 3-neck round bottom flask that was fitted with a reflux condenser and a magnetic stir bar. SPZ (3.0 g, 0.0074 mol) dissolved in 5.5 ml of hot glacial acetic acid was added to the flask, and the mixture was brought to reflux by heating the flask in an oil bath. After 35 min at reflux, removing the oil bath stopped the reaction. Thin-layer chromatography on silica gel using CH2 Cl 2 as eluent indicated the presence of a single spot, Rf 0.5, identical to that of SPZS standard and different from that of SPZ, Rf 0.0. The iron powder was allowed to settle, and then the liquid was decanted into a beaker containing 100 ml of CH2 Cl 2. The CH2 Cl 2 solution was added to a separatory funnel containing 200 ml of H2O and was shaken. After discarding the H2O layer, the CH2 Cl 2 layer was shaken with an additional 100 ml of H2O, neutralized by the dropwise addition of a solution of 30% NaOH, and shaken again. The lower CH2 Cl 2 layer was separated, dried over MgSO 4, and removed by rotary evaporation under vacuum. The oily residue (2.17 g, 0.006 mol) began to spontaneously crystallize. A few crystals were removed, and 2 ml of benzene was added to dissolve the residual material. The flask containing the product solution was immersed in a 60 to 70°C water bath up to the level of the benzene. A total of 8 ml of 95% ethanol was added with stirring, and the solution was allowed to cool to room temperature. The crystals that had been removed earlier were added to the solution; the flask was filled with argon, capped, and returned to the water bath, now at 40°C, to allow crystallization to occur under conditions of slow cooling. Mp 104 to 104.5°C uncorrected, literature 110–113°C (Pfister and Häfliger, 1961). The highly pure product was identical to authentic SPZS as confirmed by silica gel thin-layer chromatography [CHCl3 , retention time (Rt) 0.45], HPLC (C 18 reverse phase column, methanol/20 mM potassium phosphate buffer, 38:62, Rt 42.8 min), and ESI mass spectrometry, m/z 389. Residual SPZ or the presence of any other contaminant could not be detected.

Enzyme Preparations. Human liver microsomes. Human liver microsomes were prepared from human liver donors, HL120 and HL125, according to the method described by Rettie et al. (1989).

cDNA-Expressed P450s. The baculovirus-mediated expression of P450 2C9 was carried out in Trichoplusia ni insect cells according to the method of Haining et al. (1996). P450 reductase was prepared from rat liver microsomes according to the procedures described by Jones et al. (1990). Rat cytochrome b5 was a gift from Dr. K. T. Thummel (Department of Pharmacuetics, University of Washington, Seattle, WA). Incubations were conducted using a molar ratio of P450 2C9/NADPH-P450 oxidoreductase/cytochrome b5 of 1:5:1. P450 3A4 (coexpressed with reductase) was purchased from GENTEST Corporation (Woburn, MA).

Metabolic Studies with Microsomes. Microsomal incubations were con-
ducted at 37°C. Typical reaction mixtures contained 1 nmol of microsomal P450 prepared from HL120 or HL125, 50 μM substrate (SPZS or SPZ), 1 mM NADPH, and an appropriate amount of 50 mM potassium phosphate buffer, pH 7.4, in a final volume of 1 ml. The substrate and enzyme were preincubated for 4 min, and then 1 mM NADPH was added to initiate the reaction. Incubations were allowed to proceed for 30 min; they were then terminated by the addition of 4 ml of CH2Cl2 followed by vigorous vortex mixing for 20 s. Internal standard, (R)-methyl-p-tolylsulfoxide (120 μl of a 56-μg/ml buffer solution), was added to each incubation. Sodium chloride (1 g) was also added to all the incubations to saturate the aqueous layer and improve the organic solvent extractability of both the metabolites and internal standard. The resulting incubation mixtures were vortex-mixed for 60 s and centrifuged at 2000g for 15 min. The organic layer was transferred to clean tubes, and the remaining aqueous layer was extracted again with 3 ml of CH2Cl2. Combined CH2Cl2 extracts were evaporated to dryness under a gentle stream of dry nitrogen gas. The sample residue was reconstituted in 100 to 200 μl of methanol and injected directly into the HPLC column.

**HPLC Analyses.** The analyses of metabolites were performed using a SSI 220B HPLC pump system, equipped with a SSI 500 variable wavelength UV detector set at 254 nm and a C18 reverse phase column (Econosil, 5 μM, 4.6 × 250 mm, Alltech/Applied Science, State College, PA). The sample residue was dissolved in 200 μl of methanol, and 20 to 40 μl was injected into the HPLC column. For kinetic studies, an automatic injection system (Waters Associates) was used for sample injection. The metabolites were separated by isocratic elution at a flow rate of 1.3 ml/min. The mobile phase was a mixture of methanol and 20 mM potassium phosphate buffer, pH 7.0, in a ratio of 38:62.

![Fragment ions observed for SPZ under electrospray conditions and postulated mechanisms leading to their formation.](image-url)
The peak areas of metabolites and internal standard were measured and quantified using a Hewlett Packard 3394A integrator (Palo Alto, CA).

**Mass Spectral Analyses.** Electrospray mass spectra including single quadrupole mass scans and MS/MS daughter and parent ion mass scans were acquired on a Micromass Quattro II tandem quadrupole mass spectrometer (Micromass Ltd., Manchester, UK). The mass spectrometer was interfaced to two Shimadzu LC10AD solvent delivery modules and a Shimadzu LC10AV module UV-visible spectrophotometric detector monitoring the absorbance at 254 nm (Shimadzu Scientific Instruments, Inc., Columbia, MD). A cone voltage of 30 to 35 V was used for both the MS/MS daughter ion and single quadrupole mass scans, while a cone voltage of 65 V was used for the MS/MS parent ion mass scans. Argon (1 x 10^-3 mbar) was used as the collision gas at collision energy of 23 to 25 eV for both the parent and daughter ion MS/MS mass scans. The HPLC was fitted with a Beckman C18 Ultrasphere ODS column (2.0 x 250 mm), and analytes were eluted isocratically with methanol/ammonium acetate, pH 7 buffer, 50:50, as the mobile phase. Low-resolution electron impact mass spectral scans (50 – 500 amu at 4 s/decade) were acquired at 70 eV on a Micromass 70SEQ tandem hybrid mass spectrometer. Sample was introduced at 30°C via the direct insertion probe. Exact mass formula for the ions at m/z 185 and 186 found in the electrospray ionization mass spectrum of SPZ were determined on a PE Biosystems Mariner electrospray ionization-time of flight mass spectrometer fitted with a syringe pump (PE Biosystems, San Jose, CA). The pump consisted of a 5.0-ml syringe bleeding a 50:50 solution of methanol/water into the source at a rate of 40 μl/min. The sample in 5 μl of methanol was admitted to the solvent flow, from the syringe pump to the mass spectrometer, via an injection port. When the sample was detected by the mass spectrometer, ~1.3 min, 5 μl of an internal mass standard solution

![Figure 5](image)

**Figure 5.** Postulated mechanism for the formation of the ion at m/z 132 from the M + H ion of SPZO at m/z 421 via the fragment ion at m/z 251.

![Figure 6](image)

**Figure 6.** Electrospray daughter ion mass spectra of the M + H parent ion of metabolite A at m/z 405 (a) and the M + H parent ion of metabolite B at m/z 405 (b).
in acetonitrile (Hewlett Packard's ES tuning mix) was injected to confirm mass calibration of the instrument. Resolution was 4500 at 50% valley.

**Microsomal Metabolic Studies Using Modified Incubation Conditions.**

Incubations using four different sets of conditions were run in parallel. The control set of incubations was conducted as described above. The three modified sets of conditions were conducted as follows. In set 1, microsomes (HL120) in the absence of NADPH were heated to 45°C and maintained at this temperature for 5 min, before cooling to 37°C and initiating the reaction. In set 2, the pH of the incubation mixture was adjusted to 9 with a potassium phosphate buffer before reaction. In set 3, the pH was again adjusted to 9, and the detergent Lubrol PX and glycerol were added to the incubates to achieve concentrations of 0.5% w/v and 10% w/v, respectively. All incubations (1-ml final volume) contained the same amount of substrate (SPZS or SPZ, 50 μM SPZ), inhibitor, 0.5 nmol of microsomal P450, and 1 mM NADPH in a final volume of 1 ml. Control incubations contained all factors except inhibitor. Methanol was added to controls for TAO incubations. In contrast to the normal incubation procedure, FUR, DDC, and TAO were preincubicated with 0.5 nmol of microsomes and 1 mM NADPH for 10 min before addition of substrate. The work-up procedures were the same as described above, and the samples were analyzed by reverse phase HPLC.

**Metabolic Studies with cDNA-Expressed P450s. Method A.**

Incubations with cDNA-expressed P450s and 50 μM substrate (SPZS or SPZ) were performed in a manner similar to that used for microsomal incubations, except that in place of microsomes, 100 pmol of P450 3A4 Supersomes coexpressed with P450 reductase and cytochrome b5, or 100 pmol of purified CYP2C9 reconstituted with 200 pmol of P450 reductase, 100 pmol of cytochrome b5, and 10 μg of dilaurylethanolamine, were used.

**Kinetic Studies with SPZS.**

Kinetic studies were performed with cDNA-expressed P450s. Incubation procedures were the same as those described for metabolic studies, method A, except different concentrations of SPZS (5, 10, 25, 50, and 100 μM) were used, and 100 pmol of P450 2C9 was added to each incubation as the source of enzyme. Michaelis-Menten kinetic parameters for the formation of the two phenolic metabolites of SPZS (see later) were estimated initially by graphical analysis using Eadie-Hofstee plots. The parameter values obtained by this procedure were then used as parameter estimates for the nonlinear regression program SYSTAT (Wilkinson, 1987) by fitting the data to the equation: $V = V_{\text{max}} \cdot \frac{S}{(K_m + S)}$, where $V$ is the velocity.
Results

Microsomal Metabolism of SPZS and SPZ. Oxidative metabolism of SPZS and SPZ was assessed from initial in vitro studies using human liver microsomal preparations. Typical incubations were performed with a substrate concentration of 50 μM. Metabolites were quantified by reverse phase HPLC using (R)-methyl-p-tolylsulfoxide as internal standard. The HPLC profile of SPZS and its metabolites (Fig. 2a) gives a moderately intense signal for SPZ at a Rt of 21.3 min, in addition to two relatively weak signals for metabolites A and B, eluting at Rts of 14.3 min and 15.8 min, respectively. The signal intensities of SPZ and metabolites A and B (phenolic SPZs, see later) are assumed to reflect their relative quantitative importance because their relative responses to the 254-nm detecting light should not be totally different.

The HPLC profile (Fig. 2b) of the extract of a 50 μM SPZ incubation gives a moderate signal for SPZO, found at a Rt of 18.6 min. A less intense signal at a Rt of 6.8 min indicates the presence of a second much more polar metabolite, metabolite C, that elutes even earlier than the internal standard, (R)-(+)-methyl-p-tolylsulfoxide. Control incubations with SPZS and SPZ in the absence of NADPH did not produce any metabolites.

Mass Spectral Analysis. To aid the identification of metabolites A, B, and C, the mass spectrum of SPZ was determined using electrospray ionization with a cone voltage of 65 V to induce fragmentation. This spectrum was virtually identical to the daughter ion scan of the protonated parent ion of SPZ at m/z 405 shown in Fig. 3a. In addition to daughter ion scans, various mass spectral techniques such as parent ion scans, high-resolution mass measurement, and MS/MS were used to establish empirical formula and fragmentation pathways. These data in conjunction with initial sites of protonation were used to assign probable structures to the major fragment ions and possible mechanisms for their formation (Fig. 4). After initial protonation at either the sulfoxide oxygen or a nitrogen of the 5-member ring, loss of the phenylsulfoxide group yields the base peak ion at m/z 279. A second diagnostic ion resulting from initial protonation of a carbonyl oxygen occurs at m/z 211 and can be attributed to the protonated N,N-diphenylidazepirone. The substituted ketene at m/z 279 can fragment to generate the substituted pyrrolidinone at m/z 186. Alternately, it can fragment to ionized aniline at m/z 93. Parent ion scanning indicates that the ion at m/z 158 comes directly from m/z 279 or m/z 405 but not by the expulsion of CO from m/z 186, as might be expected. The base peak ion at m/z 185 is an odd electron ion having an empirical formula of C_{11}H_{7}NO_{2}. Parent ion scanning suggests that it is generated almost totally from the parent at m/z 405. The mechanism for its formation is not apparent.

A daughter ion scan of the parent M + H ion at m/z 421 for SPZO is presented in Fig. 3b. As expected, all of the ions (m/z 279, 211, 186, 185, 158, and 93) that were found to represent fragments of SPZ minus the phenylsulfoxide group in the mass spectrum of SPZ (Fig. 3a) are also present in the mass spectrum of SPZO (Fig. 3b). The major distinguishing features between the two spectra are the appearance of a base peak ion at m/z 132 for SPZO together with the lack of intensity of an ion at m/z 185, the base peak ion of SPZ. Exact mass measurements of the base peak ion at m/z 132 and an ion at m/z 251 gave an empirical formula of C_{13}H_{15}N and C_{10}H_{12}N_{2}O, respectively. The formation of the base peak ion can be rationalized as being generated from the M + H ion via the ion at m/z 251 (Fig. 5). The driving force for the intensity of this fragmentation process is presumably the ease of loss of the protonated sulfone group. The elec-
trospray mass spectrum of the HPLC peak at 18.6 min (Fig. 2b) assigned to SPZO confirmed the structural assignment, as it was identical to the mass spectrum of authentic SPZO shown in Fig. 3b.

Both metabolites A and B have M + H ions at m/z 405, suggesting that just like SPZ they are monooxidized analogs of SPZS. The mass spectrum of metabolite A (Fig. 6a) is very different from the mass spectrum of SPZ (Fig. 3a) or that of metabolite B (Fig. 6b). The absence of ions at m/z 279 and 211 coupled to the presence of corresponding ions 16 mass units higher at m/z 295 and 227 provides critical structural information. These data eliminate the thiophenyl part of the molecule as the site of hydroxylation and are only consistent with it occurring in some other residual part molecule. This conclusion is reinforced by the presence of the base peak ion at m/z 123, which can be attributed to a methylenethiophenyl ion whose formation is enhanced by the action of the highly nucleophilic sulfur atom present in the parent (Fig. 7). The moderately intense m/z 227 ion is of particular diagnostic value because its presence provides strong evidence for the metabolite having the structure of a known metabolite (2), the so called p-hydroxysulfide metabolite, in which one of the N-phenyl groups is para-hydroxylated. The absence of prominent ions at m/z 186 and 158 seen in the spectrum of SPZ (Fig. 3a), and the presence of their hydroxylated analogs at m/z 202 and 174 in metabolite A (Fig. 6a), strongly support this conclusion. However, a definitive structural assignment, i.e., whether hydroxylation is ortho, meta, or para, is not possible by mass spectrometry. Moreover, introduction of the hydroxyl group creates a second asymmetric center (saturated carbon of the pyrazodione ring plus sulfoxide) which generates diastereoisomers. Unfortunately, our HPLC conditions did not resolve the diastereoisomers. The stereochemistry of this metabolic reaction remains to be determined.

Metabolite B, Fig. 2, has prominent fragment ions at m/z 279 and 211 (Fig. 6b) similar to the mass spectrum of SPZ (Fig. 3a). These data provide compelling evidence that the phenyl group of the thiophenyl part of the molecule must be hydroxylated. The only sites available for hydroxylation are either the sulfur atom itself or the phenyl ring. Since oxidation of the sulfur atom would yield SPZ, the phenyl ring must be hydroxylated. This conclusion is reinforced by the presence of a very strong ion at m/z 139, attributable to a hydroxylated methylenethiophenophenol, by analogy to the formation of m/z 123 from metabolite A. This fragmentation is presumably driven by the presence of the nonoxidized sulfur atom in the parent (Fig. 7). Again, the specific site of hydroxylation, whether ortho, meta, or para, cannot be definitively deduced from these data alone, but precedent would suggest that it is probably para.

The M + H ion of metabolite C is found at m/z 421, suggesting that its structure is a hydroxylated analog of SPZ. The lack of prominent fragment ions at m/z 279 and 211 in the M + H daughter ion spectrum (Fig. 8) suggests that hydroxy group cannot be associated with the phenyl sulfoxide portion of the molecule or, like SPZ (Fig. 3a), these ions would be present. Therefore, the hydroxy group must be associated with the other half of the molecule. This conclusion is confirmed by the presence of prominent ions at m/z 295, 227, 202, 201, 174, and 109, all 16 mass units higher than the corresponding ions in SPZ that represent the second half of the molecule. Of particular note are the ions at m/z 109 and 227 that confine the position of hydroxylation to one of the phenyl rings of the pyrazodione ring. As with the structural assignment of metabolite A, the position of hydroxylation (ortho, meta, or para) remains in doubt, but similar to the tentative identification of metabolite A as the known p-hydroxysulfide metabolite, it can be tentatively identified as the known p-hydroxysulfinyropyrazole metabolite (Dieterle et al., 1980). The stereochemistry of this metabolic reaction remains to be determined.

**Identifying the Enzyme(s) Responsible for Oxidation of SPZS and SPZ.** Compared with control, thermal treatment of microsomes (45°C for 5 min before adding NADPH) did not significantly modify metabolite formation, i.e., SPZ, or the metabolites A and B, from SPZS (Fig. 9). However, elevating the pH to 9 decreased formation of SPZ and metabolites A and B by approximately 60% and 80%, respectively (Fig. 9). Addition of detergent (0.5% Lubrol) to the pH 9 incubation medium virtually eliminated enzyme activity (Fig. 9). Similarly, thermal treatment of microsomes did not affect metabolite formation from SPZ, but pH 9 decreased SPZO and metabolite C relative to control by 72% and greater than 95%, respectively (Fig. 10).

**Identifying the P450 Isoform(s) Responsible for Oxidation of SPZS.** Inhibition studies were used to identify the specific P450

### TABLE 1

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>$K_{m}$ (μM)</th>
<th>$V_{max}$ (nmol/nmol P450/min)</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td>16.7 (1.6)</td>
<td>2.9 (0.1)</td>
</tr>
<tr>
<td>B</td>
<td>14.3 (1.4)</td>
<td>3.5 (0.1)</td>
</tr>
</tbody>
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*The $V_{max}$ values were calculated based on the amount of internal standard, (R,S)-methyl-p-tolylsulfoxide, which was added to the incubations.*

*The numbers in parentheses are the asymptotic standard errors.*
isoform(s) responsible for the oxidative metabolism of SPZS. Specific P450 inhibitors SFZ, 50 \( \mu M \) (P450 2C9) (Miners et al., 1988); QUI, 10 \( \mu M \) (P450 2D6) (Guengerich et al., 1986); CUM, 100 \( \mu M \) (P450 2A6) (Waxman et al., 1991); DDC, 100 \( \mu M \) (P450 2E1) (Guengerich et al., 1991); TAO, 100 \( \mu M \) (P450 3A4) (Watkins et al., 1985); and FUR, 20 \( \mu M \) (P450 1A2) (Sesardic et al., 1990) were tested for their effect on the formation of metabolites. At a substrate concentration of 50 \( \mu M \), only two of the six inhibitors tested, SFZ and TAO, were effective in inhibiting the metabolism of SPZS (Fig. 11). Both dramatically decreased the formation of all three metabolites. Consistent with these results, SFZ (10 \( \mu M \)) and TAO (100 \( \mu M \)) were found to effectively inhibit the formation of SPZ and metabolites A and B at a concentration of SPZS (8 \( \mu M \)) that is close to the plasma concentration SPZS attains in vivo. Even greater inhibition was observed when the two inhibitors were used together (data not shown).

In addition to inhibiting the oxidation of SPZS to SPZ, TAO substantially inhibited (42%) the further oxidation of SPZ to SPZO (data not shown). Whether TAO also inhibited the formation of metabolite C could not be determined because of overlapping peaks at the metabolite C HPLC emergent time. SFZ, SFZ, QUI, CUM, DDC, and FUR were all ineffective in inhibiting SFZO and metabolite C formation.

**Metabolism of SPZS by cDNA-Expressed P450s.** The results of the inhibitor studies implied that P450 2C9 and 3A4 are the principal catalysts forming the oxidative metabolites of SPZS. To test this possibility directly, the catalytic activities of the cDNA-expressed forms of these specific enzymes toward SPZS were determined. In agreement with the microsomal results, both expressed P450 2C9 and P450 3A4 catalyzed the oxidation of SPZS to SPZ, although P450 3A4 was approximately 10 times more effective than P450 2C9, at least at the substrate concentration used (50 \( \mu M \) with each enzyme). Both enzymes also catalyzed the formation of metabolites A and B. In this case P450 2C9 was the more effective enzyme but by a margin much less than 10-fold.

**Metabolism of SPZ by cDNA-Expressed P450 3A4.** Analysis of the product mixture after incubation of SPZ with expressed P450 3A4 confirmed the ability of this P450 to catalyze the oxidation of SPZ to SPZO as indicated by the inhibitor studies. In addition, 3A4 was found to be responsible for the production of metabolite C.

**Stereochemistry of SPZ Formation by P450 2C9 and P450 3A4.** SPZ formed by the P450 2C9- or P450 3A4-catalyzed oxidation of SPZS was separated from the reaction mixture by HPLC using an achiral reverse phase column, and collected. SPZ was resolved into its enantiomers by reinjection of the SPZ peak back into the HPLC after refitting it with a chiral column. Under the conditions reported, the enantiomers of SPZ were virtually baseline separated and had retention times of 10.8 and 13.9 min. The prochiral stereoselectivity of both enzymes favored the enantiomer with the shortest retention time. For P450 2C9, it was 90:10 in favor of the peak at 10.8, while for P450 3A4 it was much less selective at 58:42.

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**Fig. 12.** Metabolic scheme for SPZS and SPS in human.
Kinetic Studies with cDNA-Expressed P450 2C9. Incubations with cDNA-expressed P450 2C9 were conducted at SPZS concentrations ranging from 5 to 100 μM. Eadie-Hofstee plots for the formation of both metabolite A and metabolite B were found to be linear. The kinetic parameters for metabolites A and B, obtained from nonlinear regression of the data, are summarized in Table 1. The $K_i$ values for the formation of metabolite A and metabolite B were found to be 16.7 and 14.3 μM, respectively. These values are statistically indistinguishable from that of the previously determined $K_i$ of 17 μM for the P450 2C9-catalyzed inhibition of the 7-hydroxylation of (S)-warfarin (He et al., 1995).

Discussion

A primary goal of this investigation, in addition to defining the metabolism of SPZS, was to identify the enzyme(s) responsible for reoxidation of SPZS back to SPZ. However, before studies could begin, a new supply of SPZS was needed because our supply of SPZS had become contaminated with SPZ, presumably via air oxidation. Initial attempts at purification using both column and preparative thin-layer chromatography failed to yield SPZS free of SPZ. Subsequently it was found that SPZS essentially free of SPZ could be prepared in quantitative yield by the iron in a glacial acetic acid-catalyzed reduction of SPZ.

Incubation of SPZS with human liver microsomes catalyzed the formation of SPZD and metabolites A and B. Mass spectral analyses indicated that both metabolites are monohydroxylated analogs of SPZS. The evidence is consistent with metabolite A having one of the N-phenyl groups of the pyrazolidine ring hydroxylated and with metabolite B having the phenyl group of the thiophenyl part of the molecule hydroxylated. Thus, metabolite A appears to be the known $p$-hydroxysulfide metabolite (Dieterle et al., 1980), while metabolite B appears not to have been previously reported. Mass spectral analysis of metabolite C, the polar monohydroxylated metabolite of SPZ, indicates that one of the N-phenyl groups of the pyrazolidine ring is hydroxylated. Metabolite C appears to be the known $p$-hydroxy sulfinpyrazone metabolite (Dieterle et al., 1980).

Reductive metabolism from sulfoxide to sulfide is commonly mediated by enzymes in gut flora and some tissue systems (Renwick, 1989), whereas oxidative metabolism of sulfur-containing compounds is largely dependent upon two microsomal monooxygenases: FMO and P450. To assess the relative contributions of FMO and P450 to the oxidation of SPZS and SPZ, in vitro studies were conducted using modified conditions that differentially affect the activities of FMO and P450. In general, FMO isoforms are inactivated by thermal treatment at 45°C/5 min in the absence of NADPH (Kirstein Pedersen et al., 1982), have optimal pH at 8.5 to 9 (Ziegler, 1980), and are either activated or only slightly inhibited by the presence of detergent at low concentration (Sadegh et al., 1992). In contrast, P450 isoforms show maxima at pH 7.4, are insensitive to gentle thermal treatment (45°C for 5 min), and are inactivated by low concentrations of detergent (detergent-induced dissociation of the P450/P450 reductase complex).

Metabolite formation was unaffected by thermal treatment, but it was dramatically decreased when the incubations were run at pH 9 (Figs. 9 and 10) and virtually eliminated at pH 9 in the presence of detergent (0.5% Lubrol). Clearly, P450 rather than FMO is catalyzing the oxidative metabolism of both SPZS and SPZ. These results are consistent with our recent findings that SPZS and SPZ are not substrates for human FMO3 (unpublished data). Moreover, their failure as FMO substrates is consistent with the space limitations of the FMO1 active site model proposed by Kim and Ziegler (2000). Of the P450 inhibitors tested, only SFZ and TAO were effective, indicating that P450 2C9 and P450 3A4 are the primary P450s that metabolize SPZS. This conclusion was confirmed by the formation of SPZ and metabolites A and B upon incubation of SPZS with recombinant P450 2C9 or 3A4.

Since the sulfide group of SPZ is chiral, the drug exists in two enantiomeric forms. While the commercially available form of the drug is the racemate, SPZ that is generated by microsomal enzymes need not be. Therefore, the stereoselectivity of the P450 2C9- and P450 3A4-catalyzed oxidations of SPZS to SPZ was determined by analyzing (chiral HPLC) the enantiomeric composition of SPZ obtained from each enzyme. P450 2C9 formed SPZ with a high degree of stereoselectivity, favoring the enantiomer with the shortest HPLC retention time by a ratio of 9:1. In contrast, P450 3A4 was much less stereoselective, having a ratio of about 3:2, again favoring the early emerging enantiomer. The absolute configurations of the enantiomers remain to be determined.

Given that SPZS formed in vivo from SPZ might be reoxidized to SPZ enriched in a specific enantiomer, the pharmacological properties of an administered dose of racemic SPZ could change with a changing enantiomeric ratio generated by oxidative recycling. Thus, stereoselective oxidative recycling could be problematic for relating dose to effect if the two SPZ isomers differ in either their biological properties or potencies.

The set of inhibitors described above was also used to investigate isomeric selectivity in the oxidation of SPZ to SPZO. Of the six inhibitors examined, only TAO was found to substantially inhibit the formation of SPZO (42%), indicating that it was the dominant enzyme catalyzing this metabolic reaction. This result was subsequently confirmed by incubating SPZ with recombinant P450 3A4.

The results of this study allow the construction of a partial in vivo metabolic scheme that accounts for the observed metabolic behavior of SPZ and its metabolite, SPZS, in the human (Fig. 12). In an earlier study (He et al., 1995) SPZS was found to be a potent competitive inhibitor ($K_i = 17$ μM) of P450 2C9-mediated (S)-WARF metabolism. This finding led us to conclude that SPZS was the primary determinant of the WARF-SPZ in vivo drug-drug interaction for the following reasons (He et al., 1995). The in vitro $K_i$ of SPZS for P450 2C9 is more than 10-fold lower than that of SPZ, and upon multiple dosing of SPZ, SPZS attains plasma concentrations at least equal to those of SPZ (Strong et al., 1984; Rettie et al., 1992). The findings of the present study indicate that in addition to being an inhibitor, SPZS is also a high-affinity substrate for P450 2C9 with a $K_i$ (14.3–16.7 μM; Table 1) that, within experimental error, is essentially identical to its $K_i$. Thus, the present study highlights the importance of P450 2C9 to the metabolism of both SPZS and (S)-WARF and reinforces the hypothesis that SPZS plays a critical role in the WARF-SPZ drug-drug interaction. Moreover, the projection of SPZS-based interactions to other drugs whose clearance is largely P450 2C9-dependent, such as phenytoin, tolbutamide, and diclofenac, would seem reasonable and should be anticipated. The results also indicate that the actual observation of any interaction will depend upon whether sufficiently high concentrations of SPZS can be attained, which in turn will depend upon the relative concentrations and interplay of P450 2C9, P450 3A4, and gut flora.

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