Short Communication

Nonenzymatic Formation of a Novel Hydroxylated Sulfamethoxazole Derivative in Human Liver Microsomes: Implications for Bioanalysis of Sulfamethoxazole Metabolites

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ABSTRACT:

Sulfamethoxazole is metabolized by microsomal CYP2C9 to a hydroxylamine that is thought to be responsible for the relatively high incidence of hypersensitivity reactions associated with the drug. Accurate quantification of the hydroxylamine requires the loss of metabolite through autoxidation to be blocked with ascorbate. In this study, a partly nonenzymatically generated aryl-hydroxylated derivative of sulfamethoxazole was identified by liquid chromatography/mass spectrometry in incubations of human liver microsomes, and it was found to coelute with the isomeric hydroxylamine under the conditions of three published high-performance liquid chromatography (HPLC) assays. Partial inhibition of the aryl hydroxylation by 1-aminobenzotriazole suggested some involvement of cytochrome P450. However, the formation of this compound was ascorbate-dependent, and it was enhanced by the addition of Fe²⁺/EDTA and inhibited by desferrioxamine but not by mannitol. These findings are consistent with the phenol being generated via an Fe²⁺/ascorbate/O₂-oxygenating system that does not involve hydroxyl radicals. It was also produced by H₂O₂/ascorbate. Because the compound shares close chromatographic similarities with the hydroxylamine metabolite, it is possible that previous studies may have inaccurately characterized or quantified sulfamethoxazole metabolism.

The antimicrobial sulfamethoxazole (SMX) (Fig. 1) is associated with a relatively high incidence of immune-mediated hypersensitivity reactions (Vilar et al., 2003). This is believed to be an idiosyncratic consequence of enzymatic generation of the hydroxylamine metabolite (SMX hydroxylamine; SMX-NHOH) and subsequent autoxidation to a protein-reactive nitroso species (Cribb et al., 1991). The cellular distribution (Naisbitt et al., 1999), cytotoxicity (Vyas et al., 2005; Lavergne et al., 2006), and immunogenicity (Naisbitt et al., 2001) of SMX metabolites have been studied extensively.

SMX-NHOH formation has been found both in hepatic microsomes (Cribb and Spielberg, 1990a; Cribb et al., 1995) and in vivo (Cribb and Spielberg, 1992; Gill et al., 1996). Metabolism of SMX to SMX-NHOH is catalyzed by human hepatic CYP2C9 (Cribb et al., 1995; Gill et al., 1999) and neutrophilic myeloperoxidase (Cribb et al., 1990). Although it was claimed that cyclooxygenase could also N-hydroxylate SMX, a recent study found that the oxidation of SMX in cyclooxygenase incubations containing ascorbate was due to H₂O₂ in the reaction mixture (Vyas et al., 2006). It is noteworthy that, in the context of the present observations, accurate quantification of a hydroxylamine in vitro (Cribb and Spielberg, 1990a) and in vivo (Gill et al., 1996; Winter et al., 2004) requires the loss of analyte through autoxidation to be blocked with a reducing agent; ascorbate being the agent of choice. In addition, identification of the hydroxylamine has often depended upon cochromatography with an authentic standard using relatively simple high-performance liquid chromatography (HPLC) conditions.

In this study, we identified a novel hydroxylated SMX derivative (hydroxy-SMX; SMX-OH) that is formed partly via a nonenzymatic, ascorbate-dependent pathway in microsomal incubations and is potentially a confounding factor in the quantification of SMX-NHOH. We have investigated the mechanism of its formation and determined the minimal bioanalytical requirements for accurate identification of SMX-NHOH, including a suitable chromatographic method for separation of SMX-NHOH from SMX-OH. We have also shown that SMX-OH rather than SMX-NHOH is the product of SMX’s oxidation by H₂O₂ in the presence of ascorbate.

Materials and Methods

Materials. SMX-NHOH was synthesized as described previously (Naisbitt et al., 1996), and its purity was confirmed by NMR and liquid chromatography/mass spectrometry (LC/MS) to be >99%. Unless specified otherwise, all reagents were purchased from Sigma-Aldrich (Gillingham, Dorset, UK).

Micromosomal Oxidation of SMX. Human liver microsomes (1 mg/ml) were incubated in 50 mM Tris buffer, pH 7.4, (final volume, 1 ml) at 37°C with 500 μM SMX, 1 mM ascorbate, 3.3 mM MgCl₂, and 1 mM NADPH. Other reactions additionally contained either 25 mM desferrioxamine, 0.5 M mannitol, or 1 mM 1-aminobenzotriazole. Control incubations contained SMX in the absence of either ascorbate or NADPH. After 1-h incubations, protein was precipitated by addition of an equal volume of ice-cold acetone/itrit, and supernatant was analyzed by LC/MS using one of the four LC systems listed below.

H₂O₂ Oxidation of SMX. A total of 800 μM SMX, 1 mM ascorbate, and 1 mM H₂O₂ in 50 mM Tris buffer, pH 8.0, (1 ml) were incubated at 37°C. Other reactions additionally contained either 25 mM desferrioxamine,

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ABBREVIATIONS: SMX, sulfamethoxazole; SMX-NHOH, SMX hydroxylamine; HPLC, high-performance liquid chromatography; SMX-OH, hydroxy-SMX; LC/MS, liquid chromatography/mass spectrometry; MS/MS, tandem mass spectrometry.
Results and Discussion

Identification of the Novel SMX-OH Derivative. Although standard microsomal incubations of SMX in the continuous presence of ascorbate, when they were analyzed by HPLC method 1, were found to have produced a single mass chromatogram peak corresponding to a hydroxylated metabolite ([M-H]⁻ m/z 268), this peak was also present, albeit at lower abundance, in control incubations without NADPH. Further investigations found that although the peak of m/z 268 present in these incubations coeluted with an SMX-NHOH authentic standard (Fig. 2A) under published HPLC conditions (method 1), it had a distinct tandem mass spectrometry (MS/MS) fragmentation pattern (Fig. 3) lacking the characteristic neutral loss of 17 atomic mass units seen with SMX-NHOH. In all other respects, its fragmentation corresponded to that of SMX-NHOH. Unlike the previously identified plasma and urinary metabolite 5-hydroxysulfamethoxazole (Vree et al., 1994), the neutral loss of 96 atomic mass units (loss of the 4-aminophenyl ring) was unchanged from the parent SMX molecule, demonstrating that the hydroxylation must be on the 5-methylisoxazole ring, although the precise location was not determinable. Changes to the chromatography conditions (method 4) produced good separation of the novel compound (SMX-OH) from an SMX-NHOH standard (Fig. 2B), and they also allowed an estimation of the relative abundance of the two hydroxylated metabolites by LC/MS. From the areas of their mass chromatogram peaks, it was estimated that SMX-OH was produced at approximately one third of the amount of SMX-NHOH (Fig. 4).

Neither HPLC method 2 nor method 3 resolved SMX-OH from SMX-NHOH (Fig. 2, C and D). The presence of SMX-OH in microsomal incubations without NADPH and in incubations using boiled microsomes (data not shown) suggested that it is generated...
nonenzymatically. However, the nonspecific cytochrome P450 inhibitor 1-aminobenzotriazole (Emoto et al., 2005) not only produced almost complete inhibition of the formation of SMX-NHOH in microsomes, but it also partially blocked the generation of SMX-OH (Fig. 4). Therefore, it would seem that although the microsomal production of SMX-OH requires ascorbate, the formation of SMX-OH is partly dependent on a presently uncharacterized mechanism involving cytochrome P450.

**Investigation of the Peroxidative Mechanism of SMX-OH Formation.** An incubation of SMX with H2O2 and ascorbate in Tris buffer generated a single hydroxylated metabolite with an MS/MS fragmentation pattern identical to SMX-OH. This reaction was dependent on the presence of both H2O2 and ascorbate (Table 1). Contrary to the claim of a previous study (Vyas et al., 2006), SMX-NHOH was not detected when SMX was incubated with H2O2 and ascorbate. However, the HPLC conditions (method 3) used by Vyas et al. (2006) did not separate authentic SMX-NHOH from SMX-OH when they were tested by the present authors.

The kinetics of SMX-OH formation were investigated in an SMX/
H₂O₂/ascorbate incubation. As appreciable quantities were detected within 0.1 h, it was not possible to determine the minimal required time for formation. Maximal quantities of SMX-OH were found after 1.5 h, which were stable for at least 4 h (Fig. 5). It is likely that the effective termination of the reaction after this point was due to depletion of one of the reactants, although this was not demonstrated experimentally.

Incubations of SMX with either H₂O₂ or human liver microsomes only generated SMX-OH in the presence of ascorbate. Furthermore, addition of the iron chelator desferrioxamine completely inhibited the generation of SMX-OH, whereas the hydroxyl radical scavenger mannitol (Ingelman-Sundberg et al., 1991) had no effect, even at high concentrations. Addition of FeSO₄ and EDTA to the peroxide reaction increased the amount of SMX-OH formed over 1 h (Table 1). These data are consistent with the observed aromatic hydroxylation of SMX occurring via an Fe²⁺/ascorbate/O₂ oxygenating complex (Hamilton, 1962), as first described by Udenfriend et al. (1954), in which the ascorbate acts as a complexing agent and the ultimate two-electron donor, rather than via hydroxyl radicals generated by Fenton (Mishin and Thomas, 1983) reactions. Although there have been conflicting reports regarding the formation of hydroxyl radicals by Udenfriend’s reagent (Hamilton, 1962; Ito et al., 1993; Li et al., 2003), the lack of inhibition by mannitol suggested that they were not involved here. In summary, it is concluded that locally generated H₂O₂ was not involved significantly in the ascorbate-dependent formation of SMX-OH in these microsomal incubations. The nonenzymatic hydroxylation of SMX in microsomal incubations contrasts with aromatic hydroxylation of substrates such as salicylate (Ingelman-Sundberg et al., 1991; Halliwell and Kaur, 1997) and terephthalate (Mishin and Thomas, 2004), which has been attributed to hydroxyl radicals.

Assessment of Previously Used Analytical Techniques. With very few exceptions (Cribb and Spielberg, 1990a; Cribb and Spielberg, 1990b), all previous studies to have quantified SMX-NHOH formation in microsomal or peroxidase systems, or in clinical samples, have used HPLC separation with either UV or MS detection, and identified SMX-NHOH by cochromatography with an authentic standard (Gill et al., 1996, 1999; Winter et al., 2004). We tested three published HPLC methods, designated 1, 2, and 3, for their ability to separate SMX-OH from SMX-NHOH, but none of the systems achieved this separation (Fig. 2). Furthermore, the use of either UV or simple mass detection is clearly insufficient for accurate metabolite verification, given the chromatographic similarity of SMX-NHOH to SMX-OH. The minimal requirements for accurate identification and quantification should be confirmed separation from SMX-OH and either MS-MS fragmentation analysis or conversion to nitro-SMX by addition of excess base (Rieder et al., 1988).

Whereas there is no question that the major oxidative metabolite of SMX in microsomal incubations containing NADPH is SMX-NHOH, previous studies are likely to have insufficiently separated this from the nonenzymatically generated SMX-OH, and as a result they may not have accurately identified or quantified the SMX-NHOH. These data have major implications for designing future analyses of SMX metabolite formation.

Department of Pharmacology and Therapeutics, the University of Liverpool, Liverpool, United Kingdom
(J.P.S., J.L.M., D.J.N., B.K.P.); and DMPK, GlaxoSmithKline, Ware, Hertfordshire, United Kingdom (F.J.H., S.E.C.)

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Address correspondence to: B. Kevin Park, Department of Pharmacology and Therapeutics, the University of Liverpool, Sherrington Buildings, Ashton Street, Liverpool L69 3GE, United Kingdom. E-mail: B.K.Park@liv.ac.uk