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The effect of fluconazole and ketoconazole on the metabolism of sulphamethoxazole

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1 Cytochrome P450-mediated bioactivation of sulphamethoxazole to a hydroxylamine has been implicated in the hypersensitivity reactions associated with co-trimoxazole administration. Inhibiting the formation of the hydroxylamine may be one method of preventing the high frequency of toxicity which is observed in HIV-infected patients. Therefore, in this study, we have investigated the ability of fluconazole and ketoconazole, known cytochrome P450 inhibitors, to inhibit the formation of sulphamethoxazole hydroxylamine.

2 Ten healthy male volunteers were given co-trimoxazole (800 mg sulphamethoxazole and 160 mg trimethoprim) alone or 1 h after either fluconazole (150 mg) or ketoconazole (200 mg) in a randomized fashion with a washout period of at least 1 week between each phase. Urine was collected for 24 h, and sulphamethoxazole and its metabolites were quantified by electrospray LC-MS.

3 Ketoconazole had no effect on the urinary recovery of sulphamethoxazole or any of its metabolites. In contrast, fluconazole significantly ($P < 0.001$) inhibited the formation of sulphamethoxazole hydroxylamine by $50.0 \pm 15.1\%$. Fluconazole also inhibited the oxidation of sulphamethoxazole to the 5-methylhydroxy and 5-methylhydroxy acetate metabolites by $69.9 \pm 15.8\%$ and $64.0 \pm 12.0\%$, respectively, but had no effect on the amount of sulphamethoxazole, N_4-acetyl sulphamethoxazole, or sulphamethoxazole N_1-glucuronide excreted in urine.

4 The potential clinical benefit of using fluconazole to prevent hypersensitivity to co-trimoxazole in patients with AIDS needs to be assessed in a prospective study using both metabolite formation and the clinical occurrence of adverse reactions as end-points.

Keywords sulphamethoxazole hydroxylamine toxicity metabolism inhibition fluconazole ketoconazole

Introduction

Adverse drug reactions (ADRs) associated with the use of sulphonamides have been well documented since the drugs were introduced into clinical practice in the 1930s. Co-trimoxazole, a combination of sulphamethoxazole (SMX) and trimethoprim in a ratio of 5:1, was introduced in the 1960s as a synergistic anti-bacterial combination. Over the last 30 years, a large number of idiosyncratic ADRs have been reported with co-trimoxazole including fever, rashes of variable severity, liver dysfunction and agranulocytosis [1, 2]. The sulphonamide component, SMX, has been implicated in the pathogenesis of these reactions [1]. With the advent of HIV-related disease, increased attention has focused on co-trimoxazole because it is the most effective agent for the treatment of *Pneumocystis carinii* pneumonia [3, 4], the commonest opportunistic infection in patients with AIDS [3], but causes hypersensitivity in up to 80% of the patients [5]. This compares with a frequency of 2–8% in sero-negative patients [6].

The underlying mechanism of sulphonamide toxicity

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is poorly understood. It has been suggested that the formation of a reactive intermediate from SMX is the initial step in the pathogenesis of the drug's toxicity [7]. N_4-hydroxy SMX (SMX-NOH), which is formed by oxidation of SMX, has been implicated as the reactive metabolite [8, 9]. This hydroxylamine (or a subsequent oxidation product) is thought to interact with essential cellular macromolecules (such as DNA or proteins) to initiate the tissue damage [10]. In patients who experience adverse reactions, the metabolite may not be adequately detoxified by cellular defence mechanisms [11], or alternatively, it may be produced in greater amounts [11].

The use of drugs known to inhibit the oxidative metabolism of SMX may be a way of reducing the production of reactive metabolites, thus alleviating the toxicity. This approach has been used successfully with the related compound dapsone. It has been shown that dapsone-induced methaemoglobinaemia can be inhibited *in vitro* [12] and *in vivo* in volunteers [13] and in patients on chronic therapy [14, 15]. Similarly, the toxicity of the pesticide, carbaryl, can be blocked by the administration of cimetidine [16].

The choice of enzyme inhibitor requires a knowledge of the enzyme(s) involved in the metabolism of the drug of interest. *In vitro* studies have shown that cytochrome P450 (CYP) enzymes, and in particular CYP2C9, play an important role in the formation of SMX-NOH [17]. The antifungal drugs, fluconazole and ketoconazole, which are used in HIV-infected patients, are effective inhibitors of the CYP enzymes, although the specificity of the inhibition by these two azoles may depend on the concentration of the drugs [18,19]. Fluconazole has been shown to inhibit the metabolism of phenytoin [20, 21], a CYP2C9 substrate [22], whilst ketoconazole inhibits the metabolism of cyclosporine [19, 23], a CYP3A4 substrate [24].

In the present study, we have investigated the metabolism of SMX in 10 healthy volunteers in order to determine the effect of fluconazole and ketoconazole on the formation of the metabolites of SMX, in particular SMX-NOH.

Method

Chemicals

SMX and ascorbic acid were obtained from Sigma Chemicals (Poole, UK). The internal standard, sulphadoxine (SDX), was a gift from Professor R.E. Howells (School of Tropical Medicine, Liverpool, UK). Dr A.E. Cribb (Merck Research Laboratories, West Point, PA, USA) kindly donated SMX-NOH. SMX-acetate was synthesized by a standard method for preparation of acetylated compounds using two equivalents of acetic anhydride under reflux. The remaining metabolites were gifts from Dr A.J.A.M. van der Ven (University Hospital St Radmoud, Nijmegen, Netherlands). All h.p.l.c.-grade solvents were acquired from Fisons plc (Loughborough, UK). All other chemicals were purchased from BDH Chemicals Ltd (Poole, UK).

Experimental protocol

Ethical approval was granted by the Royal Liverpool and Broadgreen University Hospital Trust. The study was conducted in 10 healthy male volunteers (mean age 32 ± 9 years, age range 21–45 years). All subjects fasted overnight, and were given in a randomized fashion either co-trimoxazole (800 mg sulphamethoxazole and 160 mg trimethoprim) alone or co-trimoxazole 1 h after ketoconazole (200 mg) or fluconazole (150 mg). Each of the three phases was separated by a washout period of at least 1 week. A higher dose of fluconazole (400 mg) was also administered to three of the volunteers. Urine was collected for 24 h into a vessel containing 1 g of ascorbic acid. The volume of urine was measured, and samples were placed in vials, frozen in liquid nitrogen, and stored at -80° C until analysed. Urine was collected for 24 h instead of 48h because (i) most of the dose (>50%) is excreted in the first 24 h [25]; and (ii) the aim of the study was to determine whether the formation of a minor metabolite could be inhibited, and thus, it was important to have a sufficient concentration of this metabolite to allow its accurate quantification.

Standard solutions of sulphamethoxazole and its metabolites

SMX, SMX-acetate (N_4-acetyl-sulphamethoxazole), 5OH-SMX acetate (N_4-acetyl-5-methylhydroxysulphamethoxazole) and the internal standard (SDX) were dissolved in h.p.l.c. mobile phase (20 mM ammonium formate (pH 2.75): acetonitrile). SMX-gluc (sulphamethoxazole N_1-glucuronide) was dissolved in water and SMX-NOH in urine containing ascorbic acid (1 mg ml^{-1}). The range of concentrations used for constructing the standard curves and the linearity achieved were as follows: SMX (50–1000 μM; $r = 0.999$), SMX-NOH (5–250 μM; $r = 0.999$), SMX-acetate (100–2000 μM; $r = 0.998$), SMX-gluc (25–750 μM; $r = 0.999$), and 5OH-SMX acetate (3–250 μM; $r = 0.997$). No calibration curve could be generated for 5OH-SMX (5-methylhydroxy-sulphamethoxazole) as insufficient standard compound was available. Therefore, the ratio of the peak areas of 5OH-SMX and the internal standard was used to compare the effect of the inhibitors.

Liquid chromatography-mass spectrometry (LC-MS) analysis of urine

Metabolites in urine were quantified by electrospray LC-MS using a Quattro II tandem quadrupole mass spectrometer, details of which have been described previously [26]. Identification was performed by co-chromatography with authentic standards. SMX, SMX-NOH, SMX-acetate, SMX-gluc, 5OH-SMX, 5OH-SMX acetate and SDX were assayed by monitoring

their pseudomolecular ions ($[M+1]^+$) at m/z 254, 270, 296, 430, 270, 312 and 311, respectively. The addition of internal standard to the urine samples and generation of calibration curves were performed on the day of analysis. SMX and SMX-acetate were quantified using a Spherex 5 C18 column (25 cm × 4.6 mm; Phenomenex, Macclesfield, Cheshire, UK). Samples were eluted with a mobile phase consisting of ammonium formate (20 mM; pH 2.75) and acetonitrile (80 : 20 v/v) at a flow rate of 1 ml min^{-1}. SMX-NOH, SMX-gluc, 5OH-SMX and 5OH-SMX acetate were quantified using a Prodigy 5 ODS 2 column (15 cm × 4.6 mm; Phenomenex) and a mobile phase of glacial acetic acid (1% v/v) and acetonitrile (80 : 20 v/v) at a flow rate of 1 ml min^{-1}. The eluate was monitored at 254 nm by a u.v.-975 absorbance detector (Jasco Corporation, Tokyo, Japan) before entering the mass spectrometer. LC-MS analysis (single ion recording) was performed under the following conditions: capillary flow of *circa* 40 µl min^{-1}, interface temperature of 60° C, capillary voltage of 4×10^3 V, and a cone voltage of 20 V. The dwell time was 2 s and the inter-channel delay was 20 ms. Data were processed via MassLynx 2 peak integration software. The limit of sensitivity of the assay for SMX-NOH, taking a coefficient of variation of ≤10% as the acceptable precision, was <1 µM; the intra-assay coefficients of variation at 10 µM, 3 µM and 1 µM were 6.0%, 3.8% and 7.4%, respectively ($n=5$). The inter-assay coefficient of variation for the measurement of SMX-NOH (30 µM) was 9.2% ($n=5$).

Statistical analysis

All the results are expressed as mean ± s.d., and as 95% confidence intervals (CI) for the difference between population means where appropriate. Data relating to urine samples from individuals administered SMX alone and from individuals administered fluconazole or ketoconazole prior to SMX were compared by the Kruskal-Wallis test. A significant difference was defined as $P<0.05$. Statistical analysis was performed using the Arcus Pro-Stat statistical software package.

Results

Urine (0–24 h) from each volunteer was initially analysed by h.p.l.c. using u.v. detection. As can be seen from Figure 1, it was not possible to separate SMX gluc, 5-OH SMX and 5OH-SMX acetate from endogenous material. This problem was overcome by LC-MS. Using single ion monitoring (Figure 2a–e) of the $[M+1]^+$ ions for SMX and each of its metabolites, satisfactory detection and resolution was achieved using identical chromatographic conditions.

The total urinary recovery (0–24 h) of SMX and its metabolites was 60.6 ± 7.0% when SMX was administered alone, and 60.0 ± 6.0% and 55.3 ± 13.8% when adminstered 1 h after ketoconazole and fluconazole, respectively. SMX underwent extensive metabolism with only 8.1 ± 4.4% (range 2.1–16.1%) of the administered dose being excreted unchanged. The major metabolite present in urine was SMX-acetate, which accounted for 39.4 ± 4.8% (range 30.5–45.0 %) of the dose. SMX-gluc and 5OH-SMX-acetate accounted for 9.3 ± 1.6% (range 7.9–13.1%) and 2.1 ± 1.6% (range 0.7–6.1%) of the administered dose, respectively. SMX-NOH was present in the urine of all ten volunteers and accounted for 1.6 ± 0.5% (range 1.0–2.6%) of the dose.

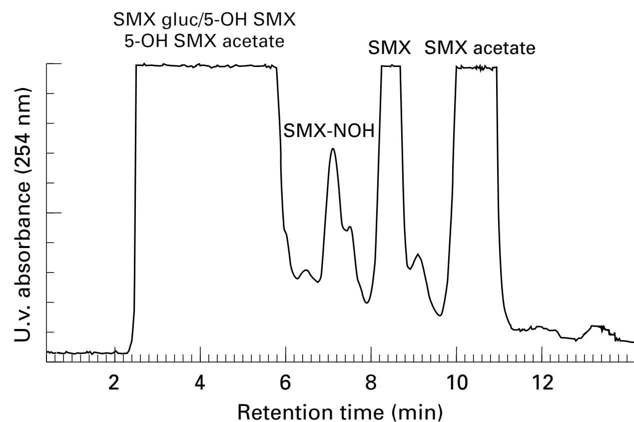

Figure 1 H.p.l.c. chromatogram of a urine sample (0–24 h) from an individual administered co-trimoxazole. SMX, SMX acetate and SMX-NOH were identified by co-chromatography with authentic standards. Elution was performed with ammonium formate–acetonitrile as described in the **Methods** section.

Ketoconazole (200 mg) did not inhibit hydroxylamine formation (95% CI for difference between population means −0.25–0.65%), or indeed any route of SMX metabolism (Figure 3). In contrast, fluconazole significantly inhibited the oxidative metabolism of SMX, affecting both N-hydroxylation and 5-hydroxylation. Fluconazole inhibited the formation of SMX-NOH by 50.0 ± 15.1% ($P<0.001$), decreasing the amount of hydroxylamine excreted from 1.6 ± 0.5% to 0.8 ± 0.2% of the dose (95% CI 0.4–1.28%). The formation of 5OH-SMX was decreased by 69.9 ± 15.8% from a peak area ratio of 1.4 ± 0.9 to 0.3 ± 0.1 (95% CI 0.3–2.1; $P<0.001$). Levels of 5OH-SMX-acetate were also decreased by 64.0 ± 12.0% from 2.1 ± 1.6% to 0.7 ± 0.5% of the administered dose (95% CI 0.54–1.23%; $P<0.001$). The higher dose of fluconazole (400 mg) which was taken by three of the volunteers had no greater effect on SMX-NOH formation (0.8 ± 0.2% ($n=3$) of the dose was excreted compared with 0.9 ± 0.3% ($n=3$) at the lower dose; $P=0.15$). Conjugation of SMX, by either acetylation or glucuronidation, was not affected by fluconazole.

Discussion

In this study we have used combined LC-MS for the detection and quantification of SMX and its metabolites in the urine of healthy volunteers. This technique offers the advantage over conventional h.p.l.c. in that non-isomeric metabolites do not require complete resolution

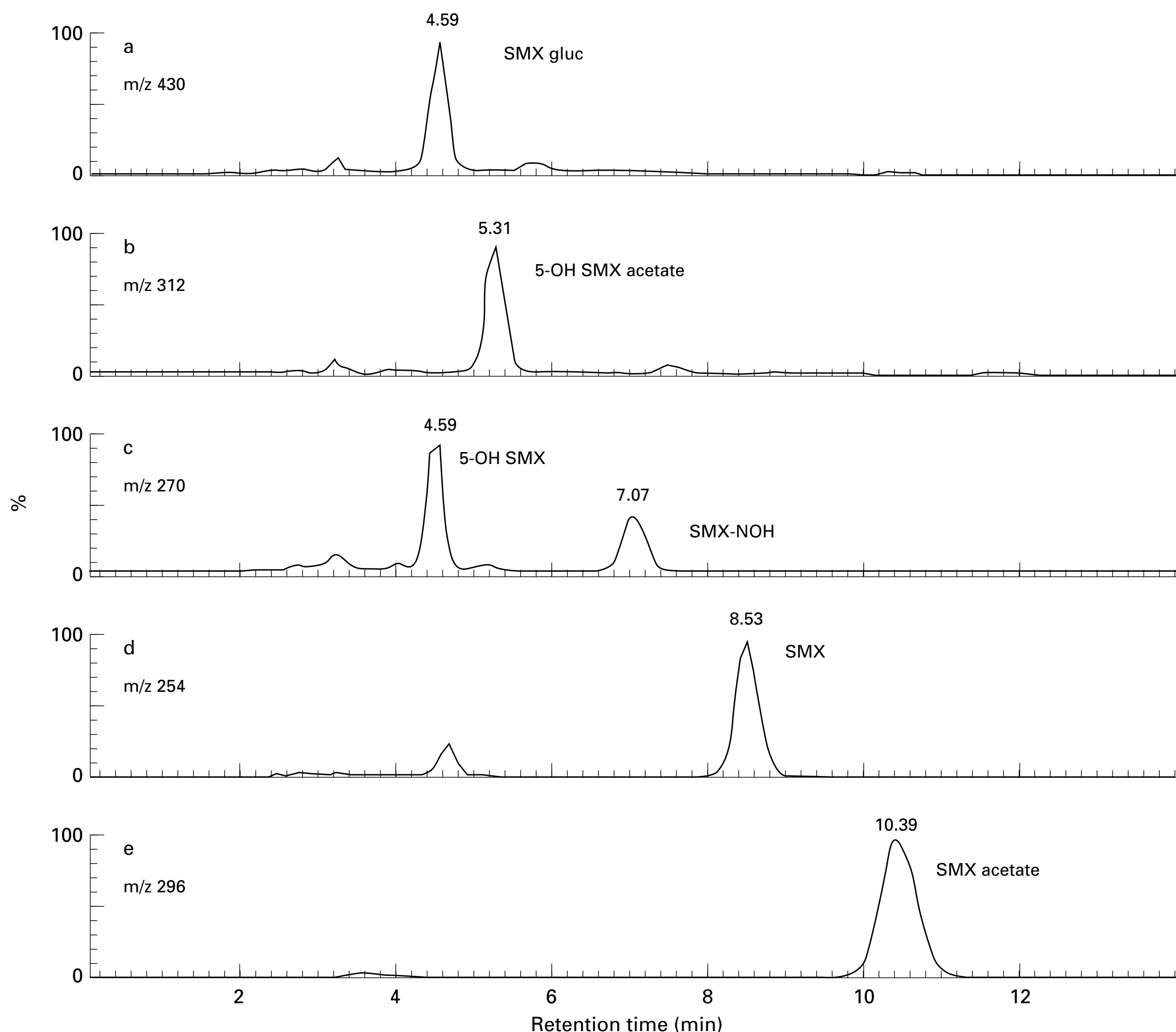

Figure 2 Single ion current chromatograms for the $[M+1]^+$ ions of SMX and its urinary metabolites. Elution was performed with ammonium formate–acetonitrile as described in the **Methods** section.

either from one another or from endogenous material. By utilizing single ion monitoring (Figure 2), the total ion current is divided into individual chromatograms which can be used to both identify and quantify the individual components present in the h.p.l.c. eluate.

SMX undergoes extensive and complex metabolism in man. In agreement with Vree *et al.* [27], we have demonstrated that up to 16% of the dose is excreted unchanged, with the N_4-acetate being the major urinary metabolite. Glucuronidation, N-hydroxylation and 5-methyl-hydroxylation accounted for the remainder of the dose excreted in human urine. In total, over 60% of the administered dose was recovered during the 24 h collection period.

Fluconazole and ketoconazole were chosen as potential inhibitors of SMX metabolism for a number of reasons. Firstly, *in vitro* studies have shown that SMX-NOH formation is dependent, at least in part, upon CYP2C9 activity [17], an enzyme inhibited by fluconazole [20, 28]. Secondly, both fluconazole and ketoconazole have been shown to inhibit the *in vivo* metabolism of a number of co-administered drugs [21, 23, 29, 30]. Thirdly, both of these drugs are already used in AIDS patients for the treatment of fungal infections [31, 32].

Consistent with *in vitro* studies with human and rat hepatic microsomes [17], fluconazole inhibited the oxidative metabolism of SMX. In all the volunteers both N-hydroxylation and 5-hydroxylation were decreased when fluconazole was pre-administered. SMX would therefore appear to be a substrate for CYP2C in both an *in vitro* microsomal system and more importantly under physiological conditions *in vivo*. Indeed, recent molecular modelling of the CYP2C active site would predict that SMX, and other sulphonamides, are substrates for this enzyme [33, 34].

Ketoconazole, which is a relatively selective inhibitor of CYP3A at low concentrations and a general CYP inhibitor at higher concentrations [18,35,36], did not inhibit SMX metabolism. This lack of inhibition may be attributed to a combination of factors. Firstly, the K_i of ketoconazole for probe substrates of CYP2C has been shown to be five times greater than fluconazole [28]. Secondly, ketoconazole is more highly protein bound than fluconazole *in vivo* [37, 38]. Thirdly, the plasma half-lives of these two inhibitors are quite

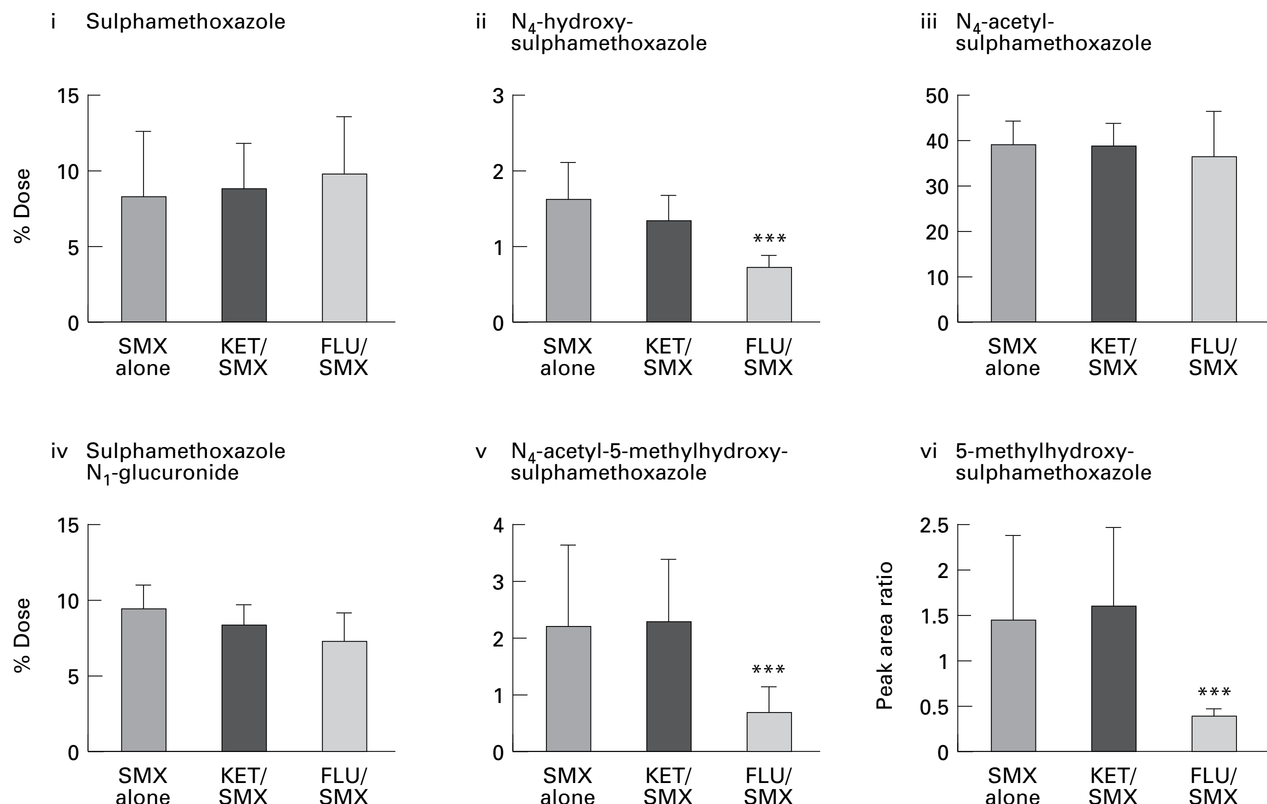

Figure 3 The effect of ketoconazole (200 mg) and fluconazole (150 mg) on the urinary excretion of SMX and its metabolites over 24 h in 10 healthy male volunteers. The data for all the metabolites are expressed as the % of dose administered, except for 5-hydroxy sulphamethoxazole, which is expressed as the peak area ratio in relation to the internal standard sulphadoxine. The data are expressed as the mean with the error bars representing the s.d. Statistical analysis was performed by the Kruskal–Wallis test by comparing the excretion of each metabolite in the absence and presence of the cytochrome P450 inhibitors, fluconazole (FLU) and ketoconazole (KET); $^{***}P<0.001$.

different, the $t_{1/2}$ of ketoconazole is *circa* 2 h whereas that of fluconazole is *circa* 30 h [39, 40]. It is also important to note that only a single dose of each inhibitor was administered, which may under-estimate the degree of inhibition that may occur on multiple dosing due to drug accumulation.

Whilst the mechanism of the idiosyncratic reactions associated with SMX (and sulphonamides in general) is poorly understood, several studies have suggested that oxidative metabolism plays an integral part in the pathogenesis. *In vitro* studies have shown that SMX is metabolized to both cytotoxic [41] and protein-reactive [42] metabolites in the presence of hepatic microsomal enzymes. These studies identified the hydroxylamine metabolite as the chemically reactive and potentially toxic species. However, more recently, further oxidation of the hydroxylamine to the nitroso metabolite has been suggested as the ultimate bioactivation step [43, 44]. In either case, the level of hydroxylamine which is formed in patients might play a pivotal role in any resultant toxicity. In the present study, SMX-NOH was identified as a urinary metabolite of SMX in all of the ten healthy volunteers and accounted for up to 2.6% of the administered dose.

Inhibition of the metabolism of SMX to the toxic species *in vivo* may provide a basis for preventing toxicity. A parallel situation has already been demonstrated with dapsone, a drug structurally related to SMX, which also undergoes CYP-mediated metabolism [26] to a toxic hydroxylamine metabolite [45]. Cimetidine, a relatively non-specific CYP inhibitor [46], decreased hydroxylamine formation and thus methaemoglobinaemia in volunteers [13] and in patients with dermatitis herpetiformis [14, 15]. Ketoconazole has also been used to inhibit the metabolism of cyclosporine in patients undergoing renal transplantation facilitating a decrease in cyclosporine dose [47].

From the present study, fluconazole rather than ketoconazole would be the more appropriate choice of drug to consider as an inhibitor of SMX metabolism and toxicity in the clinical situation. Inhibition of SMX-NOH formation by up to 50% as achieved in healthy volunteers may be of clinical importance especially if inadequate detoxication is a predisposing factor [11] in susceptible individuals. Clearly, however, this needs to be examined in HIV-infected patients using the frequency of hypersensitivity and inhibition of the formation of SMX-NOH as clinical and quantitative end points, respectively.

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