An interaction between the cytochrome P450 probe substrates chlorzoxazone (CYP2E1) and midazolam (CYP3A)

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Aims The use of multiple probe substrates to evaluate the activity of drug metabolizing enzymes requires that there are no inter-substrate interactions. As part of a series of studies to develop a clinically useful collection of probe substrates that could be given alone or in any combination, we observed an interaction between midazolam (MDZ) and another component of the six-drug cocktail. Published data indicated that the interacting component was likely to be chlorzoxazone. This was investigated as part of a second study. The data relating to the interaction from both studies are reported here.

Methods Both studies were performed in 16 healthy subjects. All treatments were given orally after an overnight fast. In study 1, which was performed to a four-period, open, crossover design, subjects received on separate occasions MDZ 5 mg, diclofenac 25 mg, a four drug cocktail (caffeine 100 mg, mephenytoin 100 mg, debrisoquine 10 mg and chlorzoxazone 250 mg) and a six drug cocktail (caffeine 100 mg, mephenytoin 100 mg, debrisoquine 10 mg, chlorzoxazone 250 mg, diclofenac 25 mg and MDZ 5 mg). In study 2, which was performed to a two-period, open, crossover design, subjects received a five drug cocktail (as the six drug cocktail in the first study, but without chlorzoxazone and with diclofenac dose increased to 50 mg) and a six drug cocktail (as five drug cocktail, with chlorzoxazone 250 mg). In both studies, blood samples were taken for measurement of plasma MDZ and 1-hydroxy MDZ (1-OH MDZ) concentrations. In study 1, blood samples were taken up to 12 h post-dose while in study 2 a single sample was taken 2 h after dosing. In study 1, the potential interaction between MDZ and the other components of the six drug cocktail was assessed by comparing AUC_{last} ratios (1-OH MDZ/MDZ) between the two treatments. Additionally, a single sampling timepoint of 2 h post-dose for determination of concentration, rather than AUC, ratios was established. The 2 h plasma concentration ratios from studies 1 and 2 were combined and a pooled analysis performed to compare ratios within each study (to determine the change in ratio when MDZ was dosed with and without chlorzoxazone) and between studies (to determine the consistency of the ratios when MDZ was given either as part of the two six drug cocktails or when given alone and as part of the five drug cocktail).

Results In study 1, both the AUC_{last} ratio and the 2 h post-dose plasma concentration ratio were reduced when MDZ was given as part of the six drug cocktail in comparison with those for MDZ alone. This was the result of an increase in MDZ, rather than decrease in 1-OH MDZ, concentrations and was considered to result from a reduction in first pass metabolism of MDZ. The geometric mean AUC_{last} values (with 95% CI) for MDZ were 95.6 (79.0, 115.7) and 160.4 (133.6, 192.6) $\mu\text{g l}^{-1} \text{ h}$ when given alone and as part of the six drug cocktail, respectively. The corresponding values for 1-OH MDZ were 789.6 (697.6, 893.6) and 791.4 (701.7, 892.6) $\mu\text{g l}^{-1} \text{ h}$. The ratio of adjusted geometric mean AUC_{last} ratios for the two treatments was 1.82 (90% CI 1.48, 2.23, $P < 0.001$). The pooled plasma 1-OH MDZ/MDZ ratio data from both studies

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showed that the differences in MDZ metabolism observed in study 1 were replicated in study 2. The adjusted geometric mean 1-OH MDZ/MDZ ratios when MDZ was given alone and as part of the six drug cocktail were 7.79 and 4.59, respectively, for study 1 (ratio 1.70, 95% CI 1.36, 2.11, $P < 0.001$) and 7.64 and 4.60 for study 2 (ratio 1.66, 95% CI 1.34, 2.06, $P < 0.001$). These data indicate that when given orally chlorzoxazone interacts with MDZ, increasing plasma MDZ concentrations. In contrast, there was no difference between the plasma 1-OH MDZ/MDZ ratios when MDZ was given alone and as part of the five drug cocktail indicating that there were no interactions between MDZ and any of the other components of that cocktail.

Conclusions Chlorzoxazone appears to significantly influence the pharmacokinetics of oral MDZ, probably through inhibition of first pass metabolism by CYP3A in the GI tract. Data from these studies and literature evidence showing a further interaction between chlorzoxazone and CYP1A2 substrates and questions concerning the specificity of chlorzoxazone as a probe substrate for CYP2E1, indicate that the use of chlorzoxazone in multisubstrate probe cocktails should be avoided.

Keywords: chlorzoxazone, CYP2E1, CYP3A, midazolam

Introduction

The activity of drug metabolizing enzymes can be assessed *in vivo* by the use of specific probe drugs. In addition to phenotyping, this approach can be used to assess the potential for a new chemical entity (NCE) to inhibit or induce specific enzyme systems and hence, in combination with *in vitro* data, provide the basis for a rational and efficient approach to a drug-drug interaction strategy. However, the administration of individual probe substrates is time consuming and, unless several experiments or large multiple arm studies are performed, may not allow the full interaction potential of an NCE to be assessed. These difficulties are exacerbated if multiple doses of the NCE are required, e.g. in cases of suspected enzyme induction. The simultaneous administration of several selective *in vivo* probes of drug metabolizing enzymes (the 'cocktail' approach) in drug development has a number of advantages. Most importantly several enzymes may be assessed in the same experiment and intrasubject variability is minimized. Before using a probe cocktail, it is essential to ensure that the cocktail components do not interact metabolically and that inclusion of multiple drugs and metabolites in a serum or urine sample does not give rise to analytical interference.

A validation study for a five-drug cocktail has been published (the 'Pittsburgh cocktail') by Frye *et al.* [1]. This cocktail includes probe substrates for CYP1A2, 2D6, 2C19, 2E1 and 3A and NAT activity. While an important step forward in the development of clinically useful multiprobe cocktails, the Pittsburgh cocktail has two limitations (which are acknowledged by the authors). First, the probe (dapsone)/metabolite combination proposed

for evaluation of CYP3A is not specific to that enzyme. At the time of publication of the Frye paper, CYP2C9 and, to a lesser extent CYP2E1, had been implicated in the biotransformation of dapsone to its hydroxylamine metabolite [2, 3]. More recent work indicates that at clinically relevant concentrations of dapsone, the enzymes primarily responsible for its hydroxylation are CYP2C8 and CYP2C9 [4]. The second limitation of the Pittsburgh cocktail was the lack of a specific probe substrate for CYP2C9.

As part of a programme to develop further the utility of the cocktail approach, we have performed two studies to evaluate a modified cocktail in which midazolam (MDZ) replaced dapsone as the CYP3A probe substrate and diclofenac was introduced as a sixth component to assess CYP2C9 activity. Data from the first study indicated that one of the components of the modified six-drug cocktail was interacting with MDZ. A review of the literature suggested that the interacting component may be chlorzoxazone, and this hypothesis was tested in a second study. The data from both studies relating to the MDZ interaction are reported here.

Methods

Both studies were performed in healthy subjects who had given written informed consent. Protocols were approved by the Glaxo Wellcome Ethics Review Committee. All subjects were nonsmokers and were not taking any medication, whether prescribed or not. Subjects were excluded if they had a regular or average alcohol consumption of more than 4 units per day. All treatments were administered orally after an overnight fast and both

studies were open label. There was a 7 day washout between treatments in both studies. The drug cocktails used were:

four drug cocktail (4-DC) comprising caffeine 100 mg, mephenytoin 100 mg, debrisoquine 10 mg and chlorzoxazone 250 mg;

five drug cocktail (5-DC) comprising caffeine 100 mg, mephenytoin 100 mg, debrisoquine 10 mg, diclofenac 50 mg and midazolam 5 mg;

six drug cocktail (6-DC) comprising caffeine 100 mg, mephenytoin 100 mg, debrisoquine 10 mg, chlorzoxazone 250 mg, diclofenac 25 mg (study 1) or 50 mg (study 2) and midazolam 5 mg.

Study 1

The study was performed in 16 healthy male subjects (mean age 27.4 years, mean height and weight 176.6 cm and 76.9 kg, respectively). There were 14 white, 1 black and 1 Asian subjects.

Each subject received the following treatments on four separate occasions: diclofenac 25 mg, MDZ 5 mg, the 4-DC and the 6-DC. Subjects were assigned to study treatment in accordance with a randomization schedule that was constrained such that the MDZ dose was always given prior to the 6-DC. This precaution was implemented to ensure the subjects' safety in a situation where a number of pharmacologically active drugs were given concomitantly. In order to minimize the number of treatment arms in the study, the published data showing that the components of the Pittsburgh cocktail did not interact were accepted. It was acknowledged that if any the four probes included from the Pittsburgh cocktail affected the metabolism of either MDZ or diclofenac (or *vice versa*), it would not be possible to determine which component was responsible.

Blood samples were taken pre-dose and 0.5, 1, 1.5, 2, 3, 4, 6, 8 and 12 h after dosing for measurement of plasma concentrations of MDZ and 1-hydroxy MDZ (1-OH MDZ). The blood was taken into lithium-heparin tubes, centrifuged within 20 min of collection at 1500 g and 4°C for 10 min and the plasma separated and stored in screw-top polypropylene sample tubes. An aliquot of each plasma sample was transferred to a separate tube and incubated with β -glucuronidase at 37°C for 3 h. All samples were frozen and stored at approximately -80°C until analysis. Samples were analysed using a validated fast gradient high performance liquid chromatography separation with tandem mass spectrometric detection [5]. The lower limits of quantification (LLOQ) for MDZ and 1-OH MDZ were 5 $\mu\text{g l}^{-1}$ and 20 $\mu\text{g l}^{-1}$, respectively. The accuracy (% bias) and precision (%CV) of the analytical method were less than 4% and 10% for both analytes at all concentrations.

Study 2

The study was performed in 16 healthy subjects (10 male and 6 female, mean age 28.4 years, mean height and weight 169.5 cm and 68.1 kg, respectively). There were 13 white and 3 black subjects. Each subject received the 5-DC and 6-DC on two separate occasions in random order. In accordance with the results of study 1, blood samples for determination of plasma MDZ and 1-OH MDZ concentrations were taken 2 h post-dose. Plasma samples were treated and analysed as described for study 1. In this study, the LLOQ for MDZ and 1-OH MDZ were 0.5 $\mu\text{g l}^{-1}$ and 2 $\mu\text{g l}^{-1}$, respectively. The accuracy (% bias) and precision (%CV) of the analytical method were less than 12% and 14% for both analytes at all concentrations.

Pharmacokinetic and statistical analyses

MDZ concentrations used in the analyses were those measured in the untreated samples whilst the 1-OH MDZ concentrations used were those measured in the β -glucuronidase treated samples. All statistical analyses were performed using SAS version 6.12 on log transformed data.

Study 1

Assessment of interaction The primary objective of the first study was to determine if there were any interactions between the components of the proposed 6-DC. The area under the plasma concentration-time curve to the last quantifiable timepoint (AUC_{last}) was calculated for MDZ and 1-OH MDZ using standard noncompartmental methods (WinNonlin, Model 200, version 1.5 using the linear/log trapezoidal rule). Geometric means and 95% CI were calculated. To assess potential interactions between MDZ and the other components of the 6-DC, the AUC_{last} 1-OH MDZ/MDZ ratios were calculated for each subject and compared between the two treatments. These were analysed using analysis of variance including terms for subject and treatment. The treatment ratio and 90% CI were estimated. A similar approach (using either AUC_{last} or Ae values, as appropriate) was used to determine if either MDZ or diclofenac had any effect on the components of the 4-DC or if there was an interaction between MDZ and diclofenac.

Assessment of single timepoint The secondary objective of the first study was to determine the most appropriate single timepoint for determination of concentration rather than AUC ratios in future studies to minimize sampling and analytical requirements. The 1-OH MDZ/MDZ plasma ratios at three timepoints (1, 2 and 4 h post dose) after the absorption phase was complete (taken as

post- $C_{\max}$) were compared with the AUC_{last} ratio. Pearson's correlation coefficients were calculated at each timepoint together with estimates of inter- and intrasubject variability.

Combined analysis of study 1 and study 2

In study 1 the 2 h post-dose plasma 1-OH MDZ/MDZ ratio was identified as an appropriate surrogate for the AUC_{last} ratio. Hence, the 2 h postdose concentration ratios were calculated for each subject in study 2 and combined with the corresponding ratios at the same timepoint from study 1. A pooled analysis was performed to compare the plasma 1-OH MDZ/MDZ ratios within each study (to determine the change in ratio when MDZ was dosed with and without chlorzoxazone) and between studies (to determine the consistency of the ratios when MDZ was given either as part of the two 6-DCs or when given alone and as part of the 5-DC). The analysis was performed using analysis of variance including terms for treatment. The treatment ratio and 90% CI were estimated.

Results

Although not reported in detail here, there was no evidence of any interaction between the components of the 6-DC other than that described below for MDZ. A summary of the results of the interaction assessments is shown in Table 1.

Study 1

The geometric mean AUC_{last} values (with 95% CI) for MDZ were 95.6 (79.0, 115.7) and 160.4 (133.6, 192.6) $\mu\text{g l}^{-1} \text{ h}$ when given alone and as part of the 6-DC,

respectively. The corresponding values for 1-OH MDZ were 789.6 (697.6, 893.6) and 791.4 (701.7, 892.6) $\mu\text{g l}^{-1} \text{ h}$. There was a statistically significant difference between the adjusted geometric mean AUC_{last} ratios for the two treatments (ratio 1.82, 90% CI 1.48, 2.23, $P < 0.001$). This indicated that one of the components of the 6-DC was influencing the pharmacokinetics of MDZ or 1-OH MDZ. Median plasma concentration plots for MDZ and 1-OH MDZ for the two treatments are shown in Figure 1.

The Pearson correlation coefficients between the plasma 1-OH MDZ/MDZ ratios at each of the three timepoints (1, 2 and 4 h post dose) and the AUC_{last} ratios, together with inter- and intrasubject variability, are shown in Table 2. The correlation values at 1, 2 and 4 h were similar for the 6-DC (0.635, 0.822 and 0.641, respectively) and following MDZ alone (0.654, 0.687 and 0.633, respectively). The 2 h sample was chosen as the most appropriate time for assessment of CYP3A activity to minimize any problems associated with sampling close to $C_{\max}$ (1 h sample) or the potential loss of data through sample concentrations falling below the LLOQ in subjects in whom CYP3A is induced (4 h sample). A scatter plot for AUC_{last} and 2 h concentration ratios is shown in Figure 2. In addition to illustrating the relationship between AUC_{last} and single point concentration ratios, the effect of administering midazolam as part of the 6-DC can be seen clearly.

Combined analysis of studies 1 and 2

The 2 h post-dose plasma 1-OH MDZ/MDZ ratios from both studies are shown as box and whisker plots in Figure 3 and the results of the combined analysis of these data are given in Table 3. The results show that the

Table 1 Results of comparison of probe substrate:metabolite ratios for each of the probes used in Study 1.

Probe substrate and metabolite	CYP	Parameter	Treatments compared	Ratio	90% CI	P value
Caffeine	1A2	AUC_{last}	4-DC/6-DC	0.99	(0.90, 1.09)	0.899
Paraxanthine						
Chlorzoxazone	2E1	AUC_{last}	4-DC/6-DC	0.87	(0.71, 1.07)	0.248
6-OH chlorzoxazone						
Debrisoquine	2D6	Ae	4-DC/6-DC	0.95	(0.71, 1.26)	0.746
4-OH debrisoquine						
Diclofenac (DCL)	2C9	Ae	DCL Alone/6-DC	1.14	(0.82, 1.58)	0.480
4-OH diclofenac						
Mephenytoin	2C19	Ae	4-DC/6-DC	1.04	(0.84, 1.28)	0.774
4-OH mephenytoin						
Midazolam	3A	AUC_{last}	MDZ Alone/6-DC	1.82	(1.48, 2.23)	<0.001
1-OH midazolam						

Parameter is the measure used in the calculation of the metabolite/probe ratio for each treatment with the exception of mephenytoin for which absolute values of urinary excretion of the 4-hydroxy metabolite were used as the assay was not stereospecific and hence concentrations of S-mephenytoin could not be measured.

Figure 1 Median plasma midazolam and 1-OH midazolam concentrations following midazolam given alone (●) and as part of the six drug cocktail (○).

changes in plasma 1-OH MDZ/MDZ ratio for the treatments with and without chlorzoxazone were very similar in both studies. The ratio (95% CI) of plasma 1-OH MDZ/MDZ ratios was 1.70 (1.36, 2.11) in the first study and 1.66 (1.34, 2.06) in the second. In both studies the difference between treatments was statistically significant ($P < 0.001$).

The between study comparison of plasma 1-OH MDZ/MDZ ratios from similar treatments showed good consistency. When MDZ was given as part of the 5-DC and alone, the adjusted geometric mean plasma 1-OH MDZ/MDZ ratios were 7.64 and 7.79, respectively, and the ratio (95% CI) was 0.98 (0.74, 1.30; $P = 0.891$). The corresponding adjusted geometric mean plasma 1-OH MDZ/MDZ ratios when MDZ was given as part of the

two 6-DC treatments were 4.60 and 4.59 and the ratio (95% CI) was 1.00 (0.76, 1.33; $P = 0.985$).

Discussion

MDZ clearance is recognized as being a marker of CYP3A activity [6] and 1-OH MDZ/MDZ plasma concentration ratios have been demonstrated to correlate with MDZ clearance [7, 8]. In the first study described here, the ratio of plasma 1-OH MDZ/MDZ concentrations 2 h after an oral dose of MDZ 5 mg was identified as a suitable single time point surrogate for AUC, changes in which should reflect changes in CYP3A activity. It should be noted that the objective of these endpoints was to detect changes in enzyme activity and not to provide estimates of absolute enzyme activity.

In Study 1, reductions were seen in both the AUC_{last} ratio and in the 2 h post-dose plasma concentration ratio when MDZ was given as part of the 6-DC in comparison with those for MDZ alone. The plasma concentration data (Figure 1) indicate that this was the result of an increase in MDZ rather than decrease in 1-OH MDZ concentrations and is likely to be the result of a reduction in first pass loss of MDZ. As MDZ is not a P-glycoprotein substrate [9], this interaction is most likely to have occurred at a metabolic rather than transporter level in the GI tract. The lack of any difference in the plasma 1-OH MDZ concentrations indicates that hepatic CYP3A was unaffected (probably due to the lower local chlorzoxazone concentrations) and may suggest a way to differentiate between GI tract and hepatic inhibition of CYP3A without recourse to oral and i.v. MDZ administration. After the clinical phase of this study was complete we became aware of data indicating that chlorzoxazone can inhibit the *in vitro* formation of 1-OH MDZ [10]. Hence it was plausible that the interaction responsible for the increased MDZ concentrations was related to the chlorzoxazone in the 6-DC. A similar interaction was not observed by Frye *et al.* during the validation of the Pittsburgh cocktail, due possibly to the high oral bioavailability of dapsone [11] coupled with its lack of specificity as a probe substrate for CYP3A [2–4].

The role of chlorzoxazone in the interaction with MDZ was investigated in study 2. The plasma 1-OH MDZ/MDZ ratio data from both studies shows clearly that the differences in MDZ metabolism observed in the first study were replicated in the second study. These data provide strong evidence that when given orally chlorzoxazone interacts with MDZ, increasing plasma MDZ concentrations. In contrast, the lack of any difference between the plasma 1-OH MDZ/MDZ ratios when MDZ was given alone and as part of the 5-DC indicates that there were no interactions between MDZ and any of the other components of that cocktail. In addition,

Table 2 Pearson correlation coefficients between 1-OH MDZ/MDZ ratios for AUC_{last} and 1, 2 and 4 h post-dose plasma concentrations.

Time	Alone	Six drug cocktail	Between subject variability	Within subject variability
1 h	0.654	0.635	0.0563	0.0602
2 h	0.687	0.822	0.0919	0.0936
4 h	0.633	0.641	0.1033	0.0553

Pearsons correlation coefficients are quoted on log transformed data.

Figure 2 Scatter plot showing the relationship between log transformed ratios (1-hydroxymidazolam/midazolam) for AUC_{last} and 2 h post-dose concentrations. ● midazolam alone, $r=0.69$; ○ midazolam in six drug cocktail, $r=0.82$.

the plasma 1-OH MDZ/MDZ ratios obtained in the presence and absence of chlorzoxazone were consistent between studies, providing reassurance that MDZ should give reproducible results when used as a probe for CYP3A as part of the 5-DC.

In addition to the interaction described between chlorzoxazone and MDZ above, an interaction between chlorzoxazone and caffeine has also been reported [12]. Such an interaction was not observed by Frye *et al.* in the Pittsburgh cocktail validation study [1]. However, the doses of chlorzoxazone and caffeine were higher in the study where an interaction was seen than in either of the studies reported here or that of Frye *et al.* [1]. The lack of any influence of chlorzoxazone on caffeine pharmacokinetics was accepted when designing the first study described here. However, had such an interaction occurred, it could not have been observed using the study design adopted. The specificity of chlorzoxazone as a probe for CYP2E1 has also been the subject of some

Figure 3 Box and Whisker plots of 2 h post-dose plasma concentration ratios (1-hydroxymidazolam/midazolam) following midazolam given with and without concomitant chlorzoxazone. Boxes show 25th, 50th and 75th percentiles and whiskers 10th and 90th percentiles with outliers plotted individually.

discussion, although current consensus appears to judge it as sufficiently specific to be useful [13].

In conclusion, chlorzoxazone appears to significantly influence the pharmacokinetics of MDZ, probably through inhibition of first pass metabolism by GI CYP3A. At higher doses than used here, chlorzoxazone has been shown to interact with caffeine and there are still questions concerning its suitability as a probe for CYP2E1. Data from these studies indicate that the use of chlorzoxazone in multisubstrate cocktails should be avoided. Although it is unfortunate that the 5-DC does not include a probe for CYP2E1, the remaining components include probes for the most commonly cited cytochrome P450 enzymes involved in drug metabolism and hence it is still of clinical utility. In addition, there is potential to expand the number of metabolic pathways examined using additional metabolite information from existing components of the 5-DC

Table 3 Comparison of plasma 1-OH MDZ/MDZ concentration ratios within and between studies.

Comparison Test/Reference	Geometric LS mean test	Geometric LS mean reference	Ratio	95% CI	P value
6-DC study 2/6-DC study 1	4.60	4.59	1.00	(0.76, 1.33)	0.985
5-DC study 2/MDZ alone study 1	7.64	7.79	0.98	(0.74, 1.30)	0.891
MDZ alone study 1/6-DC study 1	7.79	4.59	1.70	(1.36, 2.11)	<0.001
5-DC study 2/6-DC study 2	7.64	4.60	1.66	(1.34, 2.06)	<0.001

5-DC five drug cocktail, excludes chlorzoxazone.

6-DC six drug cocktail, includes chlorzoxazone.

including NAT with 5-acetylamino-6-formylamino-3-methyluracil and 1-methylxanthine formation from caffeine [14].

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