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Adult age and *ex vivo* protein binding of lorazepam, oxazepam and temazepam in healthy subjects

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WHAT IS ALREADY KNOWN ABOUT THIS SUBJECT

- The hypothesis that protein binding decreases with ageing has been used to explain studies that have not found a decline in total clearance in relation to age of highly protein bound drugs, cleared by capacity limited metabolism.
- Lorazepam, oxazepam and temazepam are highly protein bound to albumin and are cleared by capacity limited metabolism.
- There is conflicting or little data concerning the relationship between the protein binding of these drugs and age.

WHAT THIS PAPER ADDS

- In an *ex vivo* study of 60 healthy subjects (19–87 years), no clinically significant change was seen in the protein binding of these benzodiazepines with age, arguing against the hypothesis, at least in healthy subjects. The study was adequately powered to show a change of at least 7–10%.

AIM

To see if adult age correlates with *ex vivo* protein binding of lorazepam, oxazepam and temazepam in healthy subjects.

METHODS

Sixty healthy drug free subjects were recruited in the age groups 18–39, 40–64 and ≥65 years. Plasma albumin concentrations were determined. *Ex vivo* unbound fractions (f_u) were assessed by spiking samples and measuring the free and total concentrations.

RESULTS

No correlation of age with f_u was seen. The study was powered to demonstrate a change in f_u of ≥7–10%. A decline in plasma albumin concentration of ~0.03 g l^{-1} $year^{-1}$ was seen with increasing age (P = 0.032) and was associated with increased f_u of lorazepam (P = 0.009) and oxazepam (P = 0.014).

CONCLUSIONS

There was no association of adult age with *ex vivo* f_u of lorazepam, oxazepam or temazepam in healthy subjects.

Introduction

Ageing is associated with a decline in the clearance of renally cleared drugs [1] and flow-limited metabolized drugs [2, 3]. This association is less clear for capacity limited metabolized drugs, particularly those with high protein binding (low unbound fraction, f_u), in which no consistent change in total clearance (CL_{total}) with ageing has been

Table 1

Previously published *ex vivo* studies on lorazepam, oxazepam and temazepam protein binding

Drug	Cases	Mean absolute value of protein binding (f_u)	Controls	Mean absolute value of protein binding (f_u)	Change in protein binding with age
Lorazepam [5]	11, healthy, 15–73 years	0.93* (0.07)			Decreased ($P = 0.04$)
Lorazepam [13]	20, healthy, 25–86 years	0.91 (0.10)			Unchanged
Lorazepam [14]	9, healthy, mean 69 years	0.88 (0.12)	6, healthy, mean 28 years	0.90 (0.10)	Decreased ($P < 0.025$)
Male†					
Lorazepam [14]	6, healthy, mean 71 years	0.89 (0.11)	9, healthy, mean 27 years	0.89 (0.11)	Unchanged
Female†					
Lorazepam [7]	14, pre-nasal surgery, 25–86 years	*			Unchanged
Oxazepam [8]	8, healthy, mean 54 years	0.89 (0.11)	8, healthy, mean 25 years	0.87 (0.13)	Unchanged
Oxazepam [13]	20, healthy, 25–86 years	0.98 (0.02)			Unchanged
Oxazepam [9]	38, healthy, 22–84 years	0.96 (0.04)			Unchanged
Oxazepam [10]	13, healthy, 21–72 years	0.97 (0.03)			Unchanged
Temazepam [11]	18, healthy, mean 69 years	0.96–0.98*‡ (0.02–0.04)	14, healthy, mean 29 years		Decreased ($P < 0.02$)

*Absolute values of change not provided in study.
†Single study reported here twice because results analyzed by gender in study.
‡Range for entire study reported here because no mean reported in study.

found. This may be because an increase in f_u with ageing could counter a decrease in intrinsic clearance ($CL_{intrinsic}$) [4] as can be deduced from:

$$CL_{total} \approx f_u \times CL_{intrinsic}$$

The benzodiazepines lorazepam, oxazepam and temazepam are cleared by capacity limited metabolism (glucuronidation), are highly bound to albumin (low f_u) and CL_{total} appears to be unchanged with age [5–12]. There are conflicting or little data in terms of effects of ageing on protein binding, as detailed in Table 1 [5, 7–11, 13, 14]. The largest of these studies included 38 subjects. We examined a larger number of subjects to test the hypothesis that there is an increase in the f_u of these benzodiazepines with ageing.

Methods

Subjects

Sixty healthy subjects were recruited, including 10 males and 10 females in each of the groups 18–39, 40–64 and ≥ 65 years. Exclusion criteria included comorbidities (except minor degrees of dermatological, musculoskeletal or airways disease), use of regular prescription medications, use of any medications in the previous 7 days, pregnancy, current smoking, consumption of alcohol exceeding three (males) or two (females) standard drinks per day, clinical or laboratory evidence of significant hepatic or renal disease, defined as albumin ≤30 g l^{-1}, total bilirubin above the upper limit of normal, liver enzymes three times the upper limit of normal and estimated glomerular filtration rate (eGFR, [15]) ≤60 ml min^{-1} 1.73 m^{-2}. Ethics approval was obtained from the New Zealand Upper South B Regional Ethics Committee. All subjects provided written informed consent.

Protocol

A 50 ml blood sample was taken from each subject into BD Vacutainer® K_2EDTA tubes. A 5 ml aliquot was used for analysis of plasma creatinine, albumin (reported in whole numbers), total bilirubin and hepatic enzymes (Abbott Architect c8000) and plasma free fatty acids (FFA, ACS-ACOD method, Wako Pure Chemical Industries). The remaining 45 ml was centrifuged at 4°C at 4000 × *g* for 10 min and the plasma stored in 4 ml aliquots at –80°C for up to 6 months until the study day. Plasma aliquots from each subject were allowed to thaw at 23–30°C and spiked with therapeutically relevant concentrations of lorazepam 50 μg l^{-1}, oxazepam 1000 μg l^{-1} and temazepam 500 μg l^{-1} (confirmed by measurement). The samples were vortexed and incubated at 37°C for 30 min prior to being assayed.

Benzodiazepine assays

Total and free concentrations of lorazepam, oxazepam and temazepam were determined using a liquid chromatography-tandem mass spectrometry assay developed for this study [16]. Validated concentration ranges were: lorazepam (total 10–100 μg l^{-1}, free 1–10 μg l^{-1}), oxazepam (total 200–2000 μg l^{-1}, free 20–200 μg l^{-1}) and temazepam (total 100–1000 μg l^{-1}, free 10–100 μg l^{-1}) which encompassed spiked concentrations. Sample preparation consisted of plasma protein precipitation with acetonitrile to measure total concentrations, or ultrafiltration using Ultrafree-MC 30 kDa filtration devices (Millipore, Massachusetts, USA) with centrifugation at 5000 x *g* for 35 min at 23–30°C to measure free concentrations in the ultrafiltrate. Full details of this method are described in

reference [16]. No significant non-specific binding during ultrafiltration was found for the three benzodiazepines. Quality control samples (n = 6 at three concentrations) had a mean precision of <8% CV and accuracy within 94–111%. Each spiked subject sample was analyzed in triplicate, except for temazepam total concentrations, which were analyzed in duplicate. Outliers were excluded if they deviated more than 15% from the mean of the triplicates, which was only the case for 6% of the free lorazepam (11/180) and 1% of the free temazepam (2/180) concentrations. Mean CVs were ≤3.6% for the total concentrations and ≤7.4% for the free concentrations.

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics 19.0.0.1 and GraphPad Prism 5.03. The dataset was analyzed using absolute least squares linear regression analysis and Pearson's correlation coefficient, r, to express the strength of the relationships. $P < 0.05$ was considered statistically significant.

Results

The mean ages (range) for the three age groups were 30 (19–39), 50 (41–63) and 72 (65–87) years. No statistically significant differences between age groups were observed for weight, height, body mass index, lean bodyweight [17], plasma creatinine and FFA concentrations. All subjects had plasma albumin and creatinine concentrations within the normal ranges.

A slight decline in plasma albumin concentrations of 0.03 g l^{-1} $year^{-1}$ was seen with age (P = 0.032). In addition, eGFR declined with increasing age by 0.53 ml min^{-1} 1.73 m^{-2} $year^{-1}$ (P < 0.0001). The f_u of lorazepam and oxazepam, but not temazepam, increased significantly with decreasing plasma albumin concentration, with slopes (95% CI) of –0.0025 (–0.0044, –0.0007), –0.0019 (–0.0034, –0.0004) and –0.0001 (–0.0016, 0.0013) respectively.

There was no significant correlation of protein binding with age for any of the three benzodiazepines (Figure 1). This remained when gender, plasma albumin, FFA concentrations and eGFR were included as independent covariates. For the entire group, the mean f_u (95% CI) of lorazepam, oxazepam and temazepam was 0.13 (0.126, 0.133), 0.11 (0.105, 0.111) and 0.08 (0.077, 0.082) respectively.

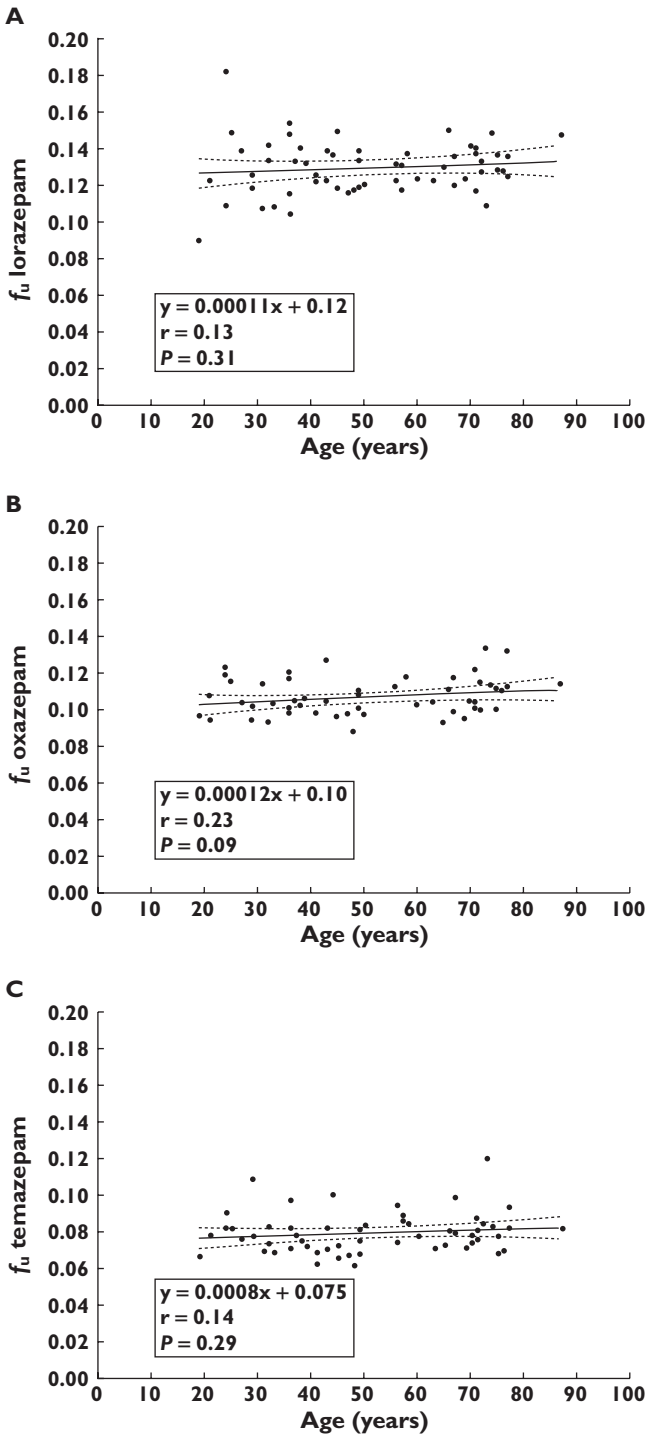

Figure 1

Mean and 95% CI from linear regression absolute least square analysis of f_u lorazepam (A), oxazepam (B) and temazepam (C) *vs.* age. Each point represents one subject. Also shown are the corresponding regression equations and Pearson's regression coefficient (r)

Discussion

We were unable to demonstrate a relationship between f_u and adult ageing for any of the three benzodiazepines examined in this group of healthy subjects who were not on medication. This is unlikely to be a power problem, as between the ages of 40 and 80 years, the minimum changes in f_u we could have detected (with 80% power and a two-tailed α of 0.05) were 8% for lorazepam, 7% for oxazepam and 10% for temazepam (Figure 1). Therefore,

an increase in f_u offsetting a decline in $CL_{intrinsic}$ with ageing is an insufficient explanation for no change in CL_{total}. For example, using our linear regression equations for lorazepam, the albumin concentration at age 80 years would need to halve in relation to that at age 40 years for the f_u to rise sufficiently to offset a 30% decline in $CL_{intrinsic}$.

Our hypothesis hinged upon the relationships between plasma albumin concentrations and age, and f_u and plasma albumin concentrations. We found that plasma albumin concentrations declined with increasing age, but the change was very small. We also demonstrated that the f_u for lorazepam and oxazepam (not temazepam), increased with decreasing plasma albumin concentrations, but again, the changes were small. It is therefore not surprising that we were unable to demonstrate a correlation between f_u and age. We are aware of three studies of these benzodiazepines that have shown a statistically significant decrease in protein binding with increasing age (Table 1). Only one study reported plasma albumin concentrations, with a decrease of 6 g l^{-1} when comparing healthy elderly and young males (mean ages 69 and 28 years respectively) [14]. The decrease of 1 g l^{-1} between our ≥65 years and <40 years groups (mean ages 72 and 30 years respectively) was much smaller.

Our absolute values of f_u of lorazepam, oxazepam and temazepam (0.13, 0.11 and 0.08 respectively), are high compared with other studies (0.09–0.12, 0.02–0.13, and 0.02–0.04 respectively) (Table 1). This may relate to the assay method used. We chose ultrafiltration as an expedient yet reliable method, whereas all the studies in Table 1 employed equilibrium dialysis. Equilibrium dialysis potentially underestimates f_u through dilution of the unbound drug by diffusion from the plasma to the buffer compartment [18].

There are a number of limitations to our study. Firstly, we selected healthy individuals as we wanted to isolate the effect of age from disease. However, ageing is associated with disease, and less restrictive exclusion criteria may have led to stronger correlations. Secondly, we investigated these drugs *ex vivo*. All blood samples were frozen for a variable amount of time before the study day, which may have led to protein degradation. The degree to which this affected our f_u results is unlikely to be significant as albumin has been shown to be stable over years of freezing [19]. Finally, protein binding was assessed in an artificial environment. The samples were left to equilibrate at 37°C but a temperature controlled centrifuge was not available meaning that the sample temperature would have dropped somewhat during ultrafiltration. The f_u of some drugs has been reported to rise with increasing temperature [20], but this is unlikely to be a significant issue in our study since previous reports used higher temperatures, and our f_u values were slightly larger. Further, pH was not controlled, which is frequently the case with ultrafiltration [21]. This may be less relevant than for equilibrium dialysis because there is no exposure to an artificial buffer during ultrafiltration.

In conclusion, we were unable to demonstrate a change in the *ex vivo* protein binding with increasing adult age of three highly protein bound, capacity-limited benzodiazepines, in spite of the study being adequately powered to show a small difference. While we found significant relationships between plasma albumin concentrations and age, and between the f_u of lorazepam and oxazepam and plasma albumin concentrations, the changes were very small. Our data suggest that changes in f_u do not provide an adequate explanation for the lack of change in CL_{total} with ageing for these benzodiazepines.

Competing Interests

There are no competing interests to declare.

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