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Serum Protein Binding of Tolterodine and its Major Metabolites in Humans and Several Animal Species[1]

Ingrid Påhlman* and Peter Gozzi
Department of Drug Metabolism, Pharmacia & Upjohn AB, SE-751 82 Uppsala, Sweden

ABSTRACT: The aim of this study was to determine *in vitro* protein binding of tolterodine and its 5-hydroxymethyl (5-HM) and *N*-dealkylated metabolites in serum from humans and several animal species at concentrations similar to those obtained in clinical and preclinical studies. Binding of tolterodine and the two metabolites to human serum albumin and α_1-acid glycoprotein (AAG) was also assessed, as was binding of tolterodine to red blood cells. *Ex vivo* protein binding of tolterodine and 5-HM was determined in serum samples from healthy volunteers treated with oral tolterodine 4 mg twice daily for 8 days. Tolterodine exhibited high protein binding in human serum; the unbound fraction (f_u) was 3.7%. The unbound fraction of tolterodine in cat and dog serum (1.5 and 2.1%, respectively) was lower compared with human serum; f_u was higher in the other species investigated (rat, 22%; mouse, 16–17%; rabbit, 39%). The unbound fraction of 5-HM was much higher in serum from humans (36%) and all animal species investigated (mouse, 72%; rabbit, 68%; cat, 32%; dog, 45%). Binding of *N*-dealkylated tolterodine to proteins in human serum was intermediate (f_u 14%). AAG was the major binding protein for tolterodine and 5-HM, and the degree of binding increased with increasing concentration of the protein. The association constant of 5-HM for AAG was lower than that of tolterodine (1.3×10^5 M^{-1} versus 2.1×10^6 M^{-1}). The blood:plasma tolterodine concentration ratio was 0.6 in both humans and dog; thus, a minor fraction of tolterodine was present in red blood cells compared with plasma (0.18 and 0.36, respectively). In the mouse, tolterodine was equally present in blood and plasma. In *ex vivo* samples, f_u values for tolterodine (pH adjusted) varied between 1.6 and 4.9% (mean 2.8%), which could be explained by differences in AAG concentrations. There was good correlation between observed f_u values for tolterodine and those predicted on the basis of AAG levels. Similar findings were observed for 5-HM.

Key words: tolterodine; protein binding; α_1-acid glycoprotein

Introduction

Tolterodine is a new muscarinic receptor antagonist intended for the treatment of overactive bladder with symptoms of urgency, increased frequency of micturition and urge incontinence. Several *in vitro* studies have shown that tolterodine has a high affinity for muscarinic receptors [1,2], while a marked inhibitory effect on bladder function has been reported following oral administration of tolterodine in healthy volunteers [3] and in patients with detrusor instability or hyperreflexia [4–6].

Tolterodine is a weak base (pK_a 9.9). In humans and several animal species, tolterodine principally undergoes two hepatic metabolic pathways: oxidation to a 5-hydroxymethyl derivative, 5-HM (lab-code DD 01; PNU-200577) and dealkylation to *N*-dealkylated tolterodine (PNU-200578), followed by further oxidation to carboxylic acids and phase II conjugates (Figure 1) [7,8]. In humans, tolterodine and 5-HM are generally almost equally present in serum, while *N*-dealkylated tolterodine is found at lower concentrations. 5-HM exerts pharmacological activity, possessing an affinity for muscarinic receptors *in vitro* similar to that of tolterodine [9], while *N*-dealkylated tolterodine was 20–40 times less active than tolterodine in functional and binding studies *in vitro*. *In vivo*, 5-HM is approximately 7-fold more potent than the parent compound in inhibiting acetylcholine-induced bladder contractions in the anaesthetized cat following intravenous administration [9]. This apparent difference in potency *in vivo* compared with the *in vitro* findings might be explained by differences in serum protein binding of the two compounds, since it is generally believed that only the unbound drug is available for pharmacological activity. Thus, the objective of this study was to determine protein binding of tolterodine and two metabolites, 5-HM and *N*-dealkylated tolterodine, in serum from humans and several animal species commonly used in preclinical pharma-

* Correspondence to: Department of Pharmacokinetics and Drug Metabolism, Astra Hässle AB, S-431 83 Mölndal, Sweden. E-mail: ingrid.pahlman@hassle.se.astra.com
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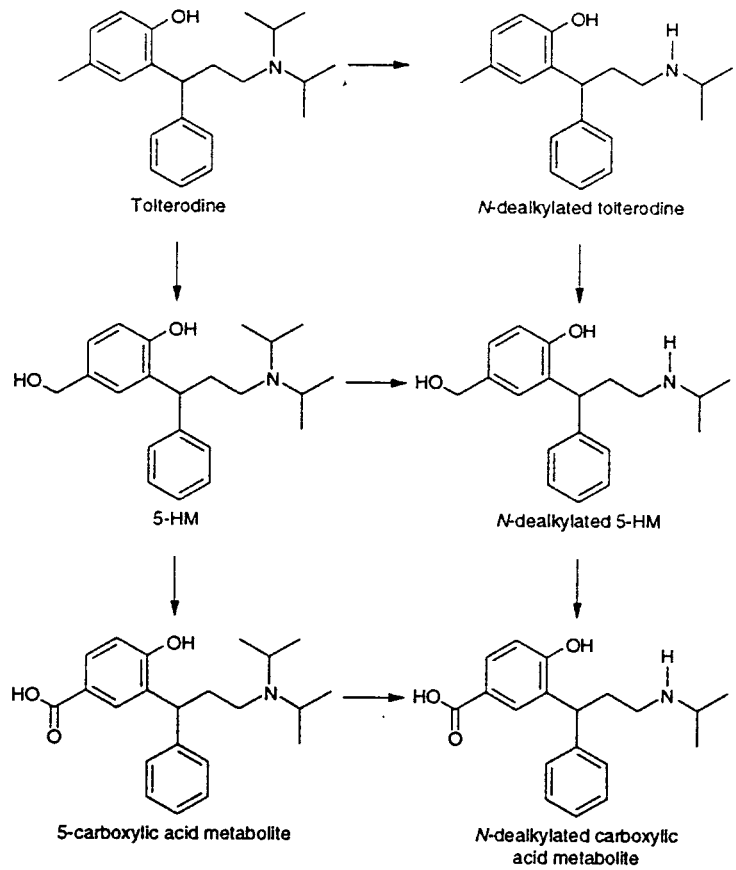

Figure 1. Metabolic pathway of tolterodine. 5-HM, 5-hydroxymethyl metabolite of tolterodine

cological and toxicological studies (rat, mouse, rabbit, cat and dog) at concentrations similar to those obtained in clinical and preclinical studies. The binding of tolterodine, 5-HM and *N*-dealkylated tolterodine to human serum albumin and α_1-acid glycoprotein (AAG; orosomucoid) was also assessed, as was binding of tolterodine to red blood cells. In addition to *in vitro* binding studies, *ex vivo* protein binding of tolterodine and 5-HM was assessed in serum samples from healthy volunteers after repeated oral administration of tolterodine.

Materials and Methods

Materials

[^{14}C]-tolterodine [(*R*)-*N*,*N*-diisopropyl-3-(2-hydroxy-5-^{14}C-methylphenyl)-3-phenylpropanamine, l-tartrate salt (PNU-200583; specific activity 4.2 MBq mg^{-1}, radiochemical purity 99.6%)], 5-HM [(*R*)-*N*,*N*-diisopropyl-3-(2-hydroxy-5-hydroxymethylphenyl)-3-phenylpropanamine, l-mandelate salt (PNU-200577)] and *N*-dealkylated tolterodine [(*R*)-*N*-isopropyl-3-(2-hydroxy-5-methylphenyl)-3-phenylpropanamine, hydrochloride salt (PNU-200578)] were provided by the Department of Medicinal Chemistry, Pharmacia & Upjohn AB, Sweden. Radiolabelled tolterodine was used without dilution with unlabelled carrier. Human serum albumin (essentially free of fatty acids), AAG, γ-globulin and 5α-androstan-3,17-dione were obtained from Sigma Chemical Company, USA, and quinaldine red indicator obtained from Merck KGaA, Germany.

Protein Solutions

Solutions of human serum albumin (45 g L^{-1}), AAG (0.75 g L^{-1}) and mixed protein solutions of AAG (0.75, 1.5 and 3.0 g L^{-1}) and human serum albumin (45 g L^{-1}) were prepared in phosphate buffer (67 mM, pH 7.4) made isotonic with NaCl. These protein concentrations were chosen to be representative of normal levels in human serum (human serum albumin, 35–50 g L^{-1}; AAG, 0.4–1.0 g L^{-1}) [10], and of increased AAG concentrations reported in patients with inflammatory disease and following trauma, respectively [11,12].

Animal and Human Serum Samples

Animal serum samples were prepared from blood taken from the following species: rat (Sprague–Dawley); mouse (NMRI and CD1); rabbit (New Zealand White); cat (European) and dog (Beagle). For each of these species, serum was collected from ≥ 5 animals and pooled prior to storage at -20°C

for ≤ 3 months. All pooled serum samples contained an equivalent volume of serum from male and female animals, with the exception of cat serum (only female serum was used).

Human serum was prepared from blood samples taken from four healthy volunteers (two males, two females) aged 30–53 years who provided oral consent prior to the study. All human serum was pooled and used on the same day as blood samples were withdrawn. Other clinical serum samples were obtained from a phase I study on tolterodine in healthy male volunteers [13]. The latter study included eight extensive and eight poor metabolizers of debrisoquine, each of whom received oral tolterodine 4 mg twice daily for 8 days. Serum for protein binding was collected 1 h after tolterodine administration on day 8. In extensive metabolizers, the tolterodine:5-HM concentration ratio varied between 0.4 and 1.5. Estimation of the unbound fraction was only possible for 5-HM, since the unbound concentrations of tolterodine were below the limit of quantitation. In poor metabolizers, tolterodine concentrations were about 7-fold higher than in extensive metabolizers allowing estimation of the unbound fraction, while 5-HM concentrations were not detectable.

Equilibrium Dialysis: In Vitro Protein Binding

Protein binding of [^{14}C]-tolterodine, 5-HM and *N*-dealkylated tolterodine was determined by means of equilibrium dialysis using Teflon cells and Spectra/Por® 2 dialysis membranes with molecular weight cutoffs of 12–14000D (Spectrum, USA). Membranes were washed in distilled water for approximately 30 min before use, a convenient method that produced the same result as when membranes were either preboiled or soaked in buffer. Serum (0.8 mL), adjusted to pH 7.4 with carbogen gas (5% CO_2, 95% O_2) and spiked with different concentrations of [^{14}C]-tolterodine, 5-HM or *N*-dealkylated tolterodine, was dialysed against an equivalent volume of phosphate buffer (67 mM, pH 7.4), made isotonic with NaCl, at 37°C for 2 h, i.e. until equilibrium was reached (according to a prestudy). To assess pH dependency, adjustments were performed to pH within the range of 7.4–8.2. All experiments were performed in triplicate. The following concentration ranges of tolterodine were investigated: rat, mouse and rabbit serum, 9–450 μg L^{-1} (28–1382 nM); cat serum, 18–450 μg L^{-1} (55–1382 nM); dog serum, 18–900 μg L^{-1} (55–2765 nM) and human serum (or protein solutions), 4.5–180 μg l^{-1} (14–553 nM). The corresponding ranges for 5-HM were: mouse, dog and cat serum, 50–500 μg L^{-1} (146–1460 nM); rabbit serum, 5–500 μg L^{-1} (15–1460 nM); and human serum (or protein solutions), 250 μg L^{-1} (5.8–146 nM). The protein binding of *N*-dealkylated tolterodine was studied in mouse serum at two concentrations (7 and 250 μg L^{-1} [25 and 879 nM]), in dog serum at 62 μg L^{-1} (218 nM) and in human serum at 15 μg L^{-1} (53 nM).

Equilibrium Dialysis: Ex Vivo Protein Binding

Equilibrium dialysis was performed on duplicate human serum samples. However, sample volumes were too small to enable pH adjustment as described in the preceding section. As an alternative, pH-adjusted observed values for the unbound fraction (f_u) were estimated from a standard curve of f_u values obtained from serum solutions of varying initial pH, dialysed against an equivalent volume of phosphate buffer (67 mM, pH 7.4), made isotonic with NaCl, at 37°C for 2 h.

Binding to Red Blood Cells

Fresh heparinized whole blood samples from humans, dog (three individuals each) and mouse (pooled sample) were, after assessing the haematocrit, incubated with [^{14}C]-tolterodine for 15 min at 37°C. The radioactivity in whole blood and the corresponding plasma was analysed after combustion in a Tri-Carb Sample Oxidizer (Packard, USA).

Serum Analysis of AAG

The AAG level in pooled animal and human serum was determined by means of a spectrofluorometric method [14], which exploits the enhanced fluorescence of quinaldine red in the presence of AAG. A standard curve was obtained in an artificial serum consisting of AAG, human serum albumin, human γ-globulins and 5α-androstan-3,17-dione in phosphate buffer. Duplicates of each serum pool were analysed. For serum samples obtained from the clinical study of Brynne *et al*. [13], AAG levels were measured at the Department of Clinical Chemistry, Huddinge University Hospital, Sweden, using a nepherometric immunoassay. All clinical samples were analysed in duplicate.

Serum Analysis of [^{14}C]-Tolterodine, Tolterodine, 5-HM and N-Dealkylated Tolterodine

The [^{14}C]-tolterodine concentration in test solutions, serum (or protein) dialysates and buffer dialysates was determined by liquid scintillation counting (Tri-Carb™ Liquid Scintillation Analyzer Model 2500 TR, Packard, USA). The results were corrected for quenching by use of an external standard. The limit of quantitation was taken as twice the background (mean 23 cpm), which equated to a limit of quantification of approximately 0.4 Bq.

Tolterodine and 5-HM concentrations in test solutions, serum (or protein) dialysates and buffer dialysates were determined using gas chroma-

Table 1. Mean (±S.D.) unbound fraction of tolterodine and its 5-hydroxymethyl and *N*-dealkylated metabolites in human serum and protein solutions

Matrix[a]	Tolterodine			5-HM			*N*-Dealkylated tolterodine		
	Concentration range (nM)	*N*	f_u (%)	Concentration range (nM)	*N*	f_u (%)	Concentration range (nM)	*N*	f_u (%)
Human serum	14–553 (4.5–180 μg L^{-1})	16	3.7 ± 0.1	5.8–146 (2–50 μg L^{-1})	14	36 ± 4	53 (15 μg L^{-1})	3	14 ± 1
HSA 45 g L^{-1}	14–553 (4.5–180 μg L^{-1})	16	58 ± 2	15–146 (5–50 μg L^{-1})	9	77 ± 7			ND
AAG 0.75 g L^{-1}	18–37 (6–12 μg L^{-1})	6	10 ± 0.7	2.9–38 (1–13 μg L^{-1})	9	80 ± 7			ND
HSA 45 g L^{-1} + AAG 0.75 g L^{-1}	14–553 (4.5–180 μg L^{-1})	18	2.7 ± 0.1	5.8–146 (2–50 μg L^{-1})	14	27 ± 4			ND
HSA 45 g L^{-1} + AAG 1.5 g L^{-1}	14–553 (4.5–180 μg L^{-1})	18	1.2 ± 0.3	29–146 (10–50 μg L^{-1})	9	14 ± 2			ND
HSA 45 g L^{-1} + AAG 3 g L^{-1}	14–553 (4.5–180 μg L^{-1})	15	0.81 ± 0.15	59–146 (20–50 μg L^{-1})	5	7.2 ± 0.4			ND

AAG, α_1-acid glycoprotein; f_u, unbound fraction; 5-HM, 5-hydroxymethyl metabolite of tolterodine; HSA, human serum albumin; *N*, number of experiments; ND, not determined.
[a] The concentration of AAG in human serum in the tolterodine and 5-HM experiments was 0.54 and 0.66 g L^{-1}, respectively.

tography-mass spectrometry, as previously described [15], while *N*-dealkylated tolterodine was measured using solid-phase extraction and liquid chromatography-mass spectrometry [16].

Evaluation of the Binding Data

The unbound fraction (f_u) of tolterodine, 5-HM or *N*-dealkylated tolterodine was calculated as follows:

$$f_u\ (\%) = \frac{C_{buffer}}{C_{serum/protein}} \times 100,$$

where C_{buffer} is the level of radioactivity or concentration in the buffer dialysate (i.e. $C_{unbound}$) and $C_{serum/protein}$ is the level of radioactivity or concentration in the serum (or protein solution) dialysate (i.e. $C_{bound} + C_{unbound}$).

Adsorption of test substance to the equipment used was negligible according to recovery experiments.

The volume shift (VS) during dialysis was estimated from the following expression:

$$VS\ (\%) = 1 - \frac{\text{Volume recovery}_{\text{buffer dialysate}}}{\text{Volume recovery}_{\text{cell average}}} \times 100.$$

Volume recovery was calculated by weighing the solutions added to the cells before dialysis, and by collecting the cell content into tared tubes after dialysis. In only 1.3% of cases (4/300) was the buffer phase diluted by more than 10%. The corresponding f_u values from these cells were omitted from further calculations.

The affinity of human serum albumin and AAG for tolterodine and 5-HM was characterized by the association constant, K_a, which was estimated by nonlinear regression analysis (WinNonlin™ version 1.1, Scientific Consulting Inc., USA) from the following expression:

$$f_u = \frac{1}{1 + K_a[nP_t - C_{bound}]}$$

where nP_t is the concentration of total binding sites. In this analysis n was set to 1, i.e. a single binding site was assumed [17]. K_a for AAG was calculated by combining binding data from experiments in mixed protein solutions and in human serum. P_t was set to the concentration of AAG, since it was considered to be the main binding protein.

The ratio of the concentration of tolterodine in red blood cells ($C_{blood\ cells}$) to that in plasma (C_{plasma}) was calculated according to the following equation:

$$C_{blood\ cells}:C_{plasma} = \frac{H - 1 + (C_{blood}/C_{plasma})}{H},$$

where H is the haematocrit and C_{blood} is the concentration of tolterodine in blood. The ratio of the concentration of tolterodine in red blood cells to that unbound in plasma ($C_{unbound,\ plasma}$) was determined as follows:

$$C_{blood\ cells}:C_{unbound,\ plasma} = \frac{(C_{blood\ cells}/C_{plasma})}{f_u}.$$

Results

Binding to Human Serum, Human Serum Albumin and AAG

Tolterodine was highly bound to protein in human serum, as indicated by a mean f_u value of 3.7%,

Table 2. Mean association constants of tolterodine and its 5-hydroxymethyl metabolite in human protein solutions

Matrix	Tolterodine			5-HM		
	P_t (μM)	K_a (M^{-1})	C.V. (%)	P_t (μM)	K_a (M^{-1})	C.V. (%)
HSA 45 g L^{-1}	669	1.1×10^3	2.0	672	$4.5 - 10^2$	14
HSA 45 g L^{-1} + AAG	13–72	2.1×10^6	1.1	16–71	1.3×10^5	3.9

C.V., coefficient of variation; K_a, association constant; P_t, total protein concentration. Other abbreviations: refer to Table 1.

while serum binding of 5-HM was about 10-fold lower (Table 1). The protein binding of *N*-dealkylated tolterodine was intermediate, with a mean f_u value of 14% (Table 1). Further investigations focused on tolterodine and 5-HM. For both compounds, the unbound fraction in human serum was independent of drug concentration. The degree of protein binding was slightly pH-dependent for tolterodine, showing approximately 30% lower f_u values in serum at pH 8.2 compared with pH 7.4 (this change in pH affects the fraction of ionized tolterodine to some extent, from approximately 90% at pH 8.2 to 99% at pH 7.4). The effect of pH on protein binding of 5-HM was negligible.

In pure protein solutions of human serum albumin and AAG, f_u did not correlate to the extent of binding in human serum, although best correlation was seen for tolterodine in AAG solutions. However, when a mixed protein solution of human serum albumin and AAG was used, f_u correlated with f_u in serum (Table 1). The proportion of unbound drug decreased progressively in the presence of increasing concentrations of AAG in the range 0.75–3.0 g L^{-1} (Table 1). Both tolterodine and 5-HM bound strongly to AAG (in the mixed protein solution) compared with pure human serum albumin, as shown by approximately 1000-fold higher K_a values for the former, although the affinity of AAG for 5-HM was approximately 10-fold lower compared with tolterodine (Table 2). K_a was calculated assuming that AAG has a single binding site, which is generally suggested [17,18].

The pH-adjusted f_u of tolterodine in *ex vivo* samples obtained from healthy volunteers who received oral tolterodine 4 mg twice daily for 8 days varied between 1.6 and 4.9% (Table 3). These variations in f_u can be explained by varying AAG concentrations in serum from different individuals (0.5–1.2 g L^{-1}). The individual *ex vivo* f_u values fitted well to the nonlinear regression of *in vitro* binding data (Figure 2). There was good correlation between observed mean f_u and the mean predicted value for both tolterodine and 5-HM, based on AAG concentrations and K_a values (Table 3). Mean observed f_u of 5-HM was 36%, similar to *in vitro* data, which was in accordance with similar AAG concentrations in the *ex vivo* serum samples and in the pooled serum used for the *in vitro* studies (0.66 g L^{-1}). In contrast, mean observed f_u of tolterodine was slightly lower compared with *in vitro* data, correlating to the lower AAG levels in pooled serum in *in vitro* studies (0.54 g L^{-1}).

Protein Binding in Animal Sera

The unbound fraction of tolterodine in animal sera varied 20-fold between different species. Compared with human serum the extent of tolterodine binding was greater in cat and dog serum, while serum binding of tolterodine in mouse, rat and rabbit serum was relatively lower (Table 4). In serum of all animal species investigated f_u for 5-HM was considerably greater than for tolterodine. As in human serum, the degree of serum protein binding of tolterodine and 5-HM was independent of drug concentration in all animal species. The ranges of AAG levels in the pooled sera of different animal species were as follows: cat, 0.27–0.38 g L^{-1}; rat, 0.23–0.32 g L^{-1}; dog, 0.37–0.60 g L^{-1}; rabbit, 0.31–0.41 g L^{-1}; and mouse, 0.99–1.1 g L^{-1}.

Binding to Red Blood Cells

The ratio of tolterodine concentration in whole blood to that in plasma was less than one (0.6) in both humans and dog, indicating low binding to red blood cells, as also shown by the smaller fractions calculated to be present in red blood cells

Table 3. Mean ± S.D. (range) unbound fraction of tolterodine (total concentration range 12–69 μg L^{-1}) and its 5-hydroxymethyl metabolite (total concentration range 1.0–9.1 μg L^{-1}) in serum samples from healthy volunteers phenotyped as poor and extensive metabolizers of debrisoquine, respectively

Parameter	Tolterodine ($n = 8$)	5-HM ($n = 8$)
AAG (g L^{-1})	0.8 ± 0.3 (0.5–1.2)	0.7 ± 0.2 (0.5–0.9)
pH	8.2 ± 0.2 (7.9–8.5)	8.2 ± 0.2 (8.0–8.5)
Observed f_u (%)	2.4 ± 1.3 (1.3–4.5)	36 ± 8 (27–49)
pH-adjusted f_u (%)	2.8 ± 1.4 (1.6–4.9)[a]	NA
Predicted f_u (%)	2.7 ± 0.9 (1.7–3.9)[b]	32 ± 5 (26–39)[c]

NA, not applicable. Other abbreviations: refer to Table 1.
[a] Calculation was based on the following expression: $f_u = -3.802[\ln(pH)] + 10.432$.
[b] Calculation based on AAG data and a K_a value of 2.1×10^6 M^{-1}.
[c] Calculation based on AAG data and a K_a value of 1.3×10^5 M^{-1}.

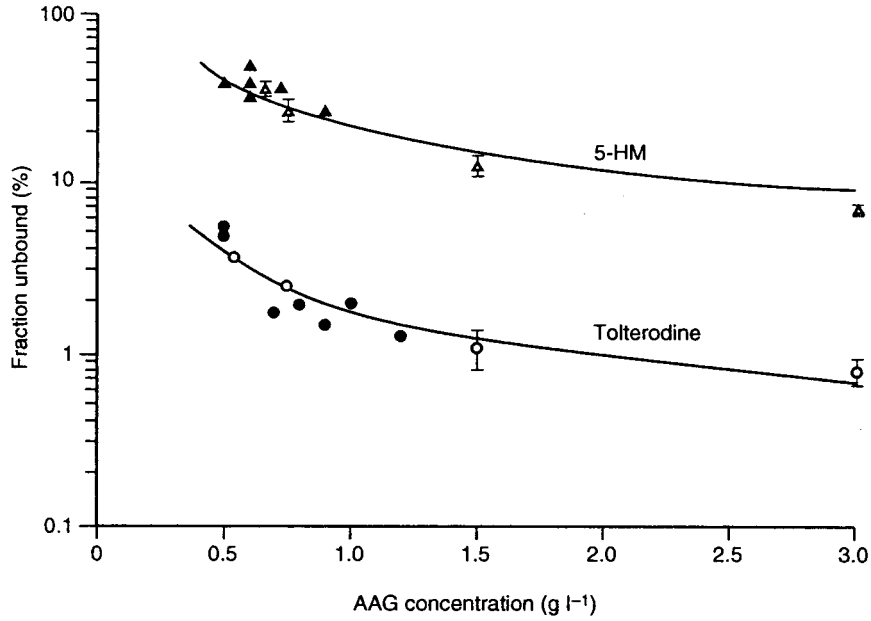

Figure 2. Observed mean (± S.D.) unbound fraction of tolterodine and its 5-hydroxymethyl metabolite (5-HM) at different concentrations of α_1-acid glycoprotein (AAG) in the presence of human serum albumin (tolterodine, ○; 5-HM, △) and *ex vivo* in healthy volunteers (tolterodine, ●; 5-HM, ▲). Solid lines indicate the nonlinear regression fit to the *in vitro* data

compared with plasma (Table 5). In the mouse, C_{blood}:C_{plasma} reached unity. Comparing concentrations of tolterodine in red blood cells to that unbound in plasma, affinity for red blood cells was comparable in humans and mouse although somewhat higher in the dog.

Discussion

In vitro, tolterodine and 5-HM bind with similar affinity to muscarinic receptors in the urinary bladder. However, the intravenous dose of drug that caused 50% inhibition of acetylcholine-induced bladder contractions *in vivo* was considerably lower for 5-HM compared with tolterodine (15 and 101 nmol kg^{-1}, respectively) [9]. The differences in serum protein binding between tolterodine and 5-HM in the cat may explain why the latter is approximately 7-fold more potent than tolterodine *in vivo*. Indeed, tolterodine was found to be $>98\%$ protein bound in cat serum, while 5-HM existed as 32% free drug. Similar findings were reported for the other species investigated, i.e. that the fraction of unbound drug in serum is considerably larger for 5-HM than for tolterodine. In human serum the unbound fraction of tolterodine was low (3.7%), while the unbound fraction of 5-HM was about 10-fold higher (36%). Generally, the serum levels of 5-HM are equal to or higher than those of tolterodine after therapeutic doses in humans [13]. As a result of the discrepancy in protein binding, it can be assumed that unbound 5-HM significantly contributes to the clinical efficacy of tolterodine. Although the protein binding of *N*-dealkylated tolterodine was intermediate in human serum (f_u 14%), this metabolite is present at much lower serum concentrations and is considerably less potent than the parent compound *in vitro*. Taken together, these findings indicate that the contribution of unbound *N*-dealkylated tolterodine to the clinical efficacy of tolterodine is negligible.

Serum protein binding of tolterodine in animals varied by about 20 times between different species, a finding that is of relevance in the evaluation of preclinical toxicology studies. For example, acute toxicity appeared in the rabbit and mouse after high doses of tolterodine yielding serum concentrations of about 370 and 770 nM, respectively, but were not apparent in the dog even after serum levels of 3100 nM (unpublished observations). Indeed, the unbound fraction of tolterodine was considerably lower in the dog (2.1%) compared with the rabbit and mouse (39 and 16–17%, respectively), resulting in higher free drug concentrations in the latter species compared with the dog. Thus, taking all these results together, it seems likely that the protein binding of tolterodine and 5-HM, and thus the concentration of unbound drug, is essential for evaluating pharmacological and toxicological activity and has important clinical implications.

In human serum, tolterodine and 5-HM appear to be bound predominantly to AAG rather than human serum albumin. AAG plays a major role in the binding of several basic drugs, including chlorpromazine, imipramine, lidocaine, propranolol, quinine and triazolam [12,19–25]. AAG is an acute phase protein whose concentration increases during several conditions such as stress, infection, inflammation, cancer and surgery, and for several drugs that

Table 4. Mean (± S.D.) unbound fraction of tolterodine and its 5-hydroxymethyl and *N*-dealkylated metabolites in serum of several animal species

Species	Tolterodine			5-HM			*N*-Dealkylated tolterodine		
	Concentration range (nM)	*N*	f_u (%)	Concentration range (nM)	*N*	f_u (%)	Concentration range (nM)	*N*	f_u (%)
Rat	28–382 (9–50 μg L^{-1})	9	22 ± 2			ND			ND
Mouse									
NMRI strain	28–382 (9–50 μg L^{-1})	9	16 ± 2	146–1460 (50–500 μg L^{-1})	6	72 ± 13			ND
CD1 strain	305–1535 (100–500 μg L^{-1})	6	17 ± 0.01			ND	25–879 (7–250 μg L^{-1})	5	16 ± 2
Rabbit	28–1382 (9–450 μg L^{-1})	9	39 ± 1	15–1460 (5–500 μg L^{-1})	9	68 ± 6			ND
Cat	55–1382 (18–450 μg L^{-1})	9	1.5 ± 0.2	146–1460 (50–500 μg L^{-1})	6	32 ± 4			ND
Dog	55–2765 (18–900 μg L^{-1})	8	2.1 ± 0.2	146–1460 (50–500 μg L^{-1})	6	45 ± 7	218 (62 μg L^{-1})	3	3.8 ± 0.3

Abbreviations: refer to Table 1.

bind to this protein there is a clearly described relationship between AAG concentration and plasma protein binding [12,20,21,23]. This was also the case for tolterodine and 5-HM, although the affinity of 5-HM for AAG was approximately 10-fold lower. Negative correlation was evident between the unbound concentration of tolterodine and 5-HM and the concentration of AAG. Within a small population of healthy volunteers, the unbound fraction of tolterodine and 5-HM varied by 3.5- and 2-fold, respectively [13]. In patients, however, it can be expected that f_u will be subject to larger variation.

The binding of tolterodine and 5-HM in a pure protein solution of AAG did not predict binding in serum. However, when AAG was mixed with human serum albumin the unbound fraction was similar to that in serum. Similar protein interaction leading to increased binding to AAG has been reported for other substances [26–28]. In addition, correlation between AAG concentration and f_u *in vitro* fitted well to the observed f_u values *ex vivo* in human serum. The 1000-fold higher association constant for AAG mixed with human serum albumin compared with pure human serum albumin further support that AAG is the major binding protein for tolterodine and 5-HM.

The good correlation between protein binding of tolterodine and AAG concentration allowed prediction of the AAG concentration or f_u values by using the association constant derived from nonlinear regression analysis. Although differences in serum AAG concentration may explain variations in the unbound fraction of tolterodine in the small population of healthy volunteers studied, the large interspecies differences in f_u could not be explained by differences in AAG concentrations. Indeed, analyses of AAG levels in the pooled sera used in the *in vitro* protein binding experiments showed similar values in the rat, rabbit, cat and dog, while AAG levels were considerably elevated in the mouse. Genetic interspecies variation in AAG may explain these findings. In humans, for example, three genetic variants of AAG have been described (F1, S and A) that differ in terms of their drug-binding properties [29]. The possible clinical consequences of differences in tolterodine and 5-HM binding between individuals with different AAG phenotypes remains to be elucidated.

Another important consideration for a drug that is highly protein bound is the potential for interaction

Table 5. Mean (± S.D.) *in vitro* concentration ratio of tolterodine in whole blood or red blood cells to that in plasma (total or unbound concentration) in humans, dog and mouse

Species	*H* (%)	C_{blood}:C_{plasma}	$C_{blood\ cells}$:C_{plasma}	$C_{blood\ cells}$:$C_{unbound,\ plasma}$
Humans ($n = 3$)	44.6 ± 3.5	0.64 ± 0.02	0.18 ± 0.03	5.0 ± 0.8
Dog ($n = 3$)	50.7 ± 4.6	0.63 ± 0.06	0.36 ± 0.08	17 ± 4
Mouse (pooled blood)	43.8	1.07 ± 0.05[a]	1.2	7.2

C_{blood}, blood concentration; $C_{blood\ cells}$, concentration in red blood cells; C_{plasma}, total plasma concentration; $C_{unbound,\ plasma}$, unbound plasma concentration; *H*, haematocrit.
[a] Mean (± S.D.) of four separate assays of the pooled sample.

with other drugs that exhibit a high affinity for serum proteins. Competition for the same binding site may lead to increases in the unbound concentration of one of the drugs in serum, thus enhancing its therapeutic effect, producing unexpected toxicity. Indeed, other basic AAG-binding drugs, including alprenolol, chlorpromazine, imipramine, propranolol and verapamil, did increase the unbound fraction of tolterodine by 20–33% at equimolar concentrations of drug and protein (unpublished observations). However, no significant interaction was seen at therapeutic concentrations. For tolterodine to displace other drugs from AAG its serum concentration during therapy must approach or exceed 9–23 μM [10], the molar concentration of AAG in serum. This is approximately 1000-fold higher than the peak serum concentration of tolterodine observed following an oral dose of tolterodine 3.2 mg (19 nM) [30]. Since the optimal dosage of tolterodine for the treatment of overactive bladder symptoms appears to be 1–2 mg twice daily [31], it is unlikely that concomitant administration of tolterodine will lead to significant displacement of basic drugs from AAG. However, the potential remains for tolterodine to be displaced from AAG by other drugs with a small volume of distribution, since the molar concentration of AAG in serum is relatively low.

In summary, tolterodine is highly protein bound in serum from humans, cats and dogs, while serum binding is lower in other species (mouse, rabbit, rat). In contrast to tolterodine, the fraction of unbound drug in serum is larger for 5-HM in human serum and in sera from all animal species included in this study. Binding of *N*-dealkylated tolterodine in human serum is intermediate between that of tolterodine and 5-HM. In humans, tolterodine and 5-HM bind to AAG in preference to human serum albumin, while 5-HM shows a lower affinity for AAG than tolterodine. The differences in protein binding between tolterodine and 5-HM may explain why the latter appears to possess increased pharmacological activity *in vivo*.

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