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# Muscarinic receptor binding, plasma concentration and inhibition of salivation after oral administration of a novel antimuscarinic agent, solifenacin succinate in mice

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**1** A novel muscarinic receptor antagonist, solifenacin succinate, inhibited specific binding of [*N*-methyl-$^3$H]-scopolamine ([$^3$H]-NMS) in the mouse bladder, submaxillary gland and heart in a concentration-dependent manner. This inhibitory effect was greatest in the submaxillary gland, followed by the bladder and heart.

**2** After oral administration of oxybutynin (76.1 $\mu$mol kg$^{-1}$) or solifenacin (62.4, 208 $\mu$mol kg$^{-1}$), a significant dose- and time-dependent increase in $K_D$ values for specific [$^3$H]-NMS binding was seen in the bladder, prostate, submaxillary gland, heart, colon and lung, compared with control values. The increase in $K_D$ induced by oxybutynin in each tissue reached a maximum 0.5 h after oral administration and then rapidly declined, while that induced by solifenacin was greatest 2 h after administration and was maintained for at least 6 or 12 h, depending on the dose. The muscarinic receptor binding of oral solifenacin was slower in onset and of a longer duration than that of oxybutynin.

**3** Plasma concentrations of oxybutynin and its active metabolite (*N*-desethyl-oxybutynin, DEOB) were maximum 0.5 h after its oral administration and then declined rapidly. Oral solifenacin persisted in the blood for longer than oxybutynin.

**4** Pilocarpine-induced salivary secretion in mice was significantly reduced by oral administration of solifenacin and was completely abolished 0.5 h after oral oxybutynin. Although the suppression induced by solifenacin was more persistent than that due to oxybutynin, the antagonistic effect of solifenacin on the dose–response curves to pilocarpine was significantly weaker than that of oxybutynin. It is concluded that oral solifenacin persistently binds to muscarinic receptors in tissues expressing the $M_3$ subtype, such as the bladder.
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**Keywords:** Bladder; submaxillary gland; oxybutynin; solifenacin; muscarinic receptor binding; plasma concentration; salivary secretion

**Abbreviations:** $B_{max}$, maximum number of binding sites; DEOB, *N*-desethyl-oxybutynin; [$^3$H]-NMS, [*N*-methyl-$^3$H]-scopolamine methyl chloride; $K_D$, apparent dissociation constant; $K_i$, inhibition constant

## Introduction

An overactive bladder is characterized by symptoms of increased frequency of micturition, urgency and urge incontinence (Bulmer & Abrams, 2000; Abrams *et al*., 2002). Muscarinic receptor antagonists such as oxybutynin have been used for some time to treat overactive bladder (Andersson, 1988; Wein, 1990). However, the oral use of oxybutynin is often limited by frequent and serious systemic side effects such as dry mouth, blurred vision, constipation and tachycardia (Yarker *et al*., 1995). To reduce or even eliminate this problem, novel antimuscarinic agents that exhibit pharmacological selectivity in the bladder relative to other tissues such as the salivary gland have been developed (Nilvebrant *et al*., 1997; Abrams *et al*., 1998; Anderson *et al*., 1999; Gupta & Sathyan, 1999; Chapple, 2000).

Muscarinic cholinoceptors have been classified into five subtypes ($M_1$–$M_5$) based on genetic and pharmacological characteristics (Hulme *et al*., 1990; Caulfield, 1993). Although both $M_2$ and $M_3$ subtypes coexist in smooth muscle, the $M_2$ subtype predominates but functional *in vitro* data with a number of selective antagonists indicate that the contraction of most smooth muscle, including the urinary bladder, is mediated by the $M_3$ subtype (Caulfield, 1993; Eglen *et al*., 1994). On the basis of these results, muscarinic receptor antagonists with a higher affinity for $M_3$ than $M_2$ subtypes should be more beneficial in the treatment of overactive bladder.

Solifenacine succinate (YM905; [(+)-(1*S*,3′*R*)-quinuclidin-3′-yl-1-phenyl-1,2,3,4-tetrahydroisoquinoline-2-carboxylate monosuccinate]) is a novel muscarinic receptor antagonist developed for the treatment of urinary incontinence and other symptoms of overactive bladder (Ikeda *et al*., 2002; Kobayashi *et al*., 2004). *In vitro* radioligand studies with human recombinant

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muscarinic subtypes have revealed that solifenacin exhibits high affinity and specificity for the muscarinic $M_3$ subtype relative to the $M_1$ and $M_2$ subtypes; the affinity of solifenacin for these subtypes is 6–10 times less than that of oxybutynin (Ikeda *et al*., 2002). Solifenacin has been found to be equipotent with oxybutynin at inhibiting carbachol-stimulated $Ca^{2+}$ mobilization in detrusor cells, but less potent in submandibular gland cells (Ikeda *et al*., 2002; Kobayashi *et al*., 2004). *In vivo* studies in anaesthetized rats have shown that solifenacin, unlike oxybutynin, is more potent at inhibiting bladder contraction than salivation (Ikeda *et al*., 2002; Ohtake *et al*., 2004).

A number of authors have indicated the importance of characterizing the binding of ligands to receptors in the presence of various pharmacokinetic and pharmacodynamic factors (Beauchamp *et al*., 1995; Uchida *et al*., 1995; Ohkura *et al*., 1998; Yamada *et al*., 2001; 2002; 2003). The aim of the present study was to characterize the *in vivo* muscarinic receptor binding properties of solifenacin (after oral administration) in the bladder and submaxillary gland, compare them with those of oxybutynin and relate them to their plasma levels and inhibitory effects on salivary secretion.

## Methods

### *Animals*

Male ddY strain mice aged 11 to 16 weeks (Japan SLC Inc., Shizuoka, Japan) were used. They were housed under a 12-h light–dark cycle and fed laboratory chow and water *ad libitum*.

### *Administration of oxybutynin and solifenacin*

Mice were fasted for 16 h, then orally administered oxybutynin (76.1 $\mu$mol kg$^{-1}$) or solifenacin (62.4 or 208 $\mu$mol kg$^{-1}$) dissolved in distilled water. Control animals received vehicle alone. The study was conducted in accordance with the guidelines of the Experimental Animal Ethical Committee of the University of Shizuoka.

### *Tissue preparation*

At 0.5 to 24 h after drug administration, the mice were anaesthetized with diethyl ether and exsanguinated by taking the blood from the descending aorta. The tissues were then perfused with cold saline *via* the aorta. The bladder, prostate, submaxillary gland, heart, lung and colon were dissected out, and any fat and blood vessels removed. As the individual organs did not weigh very much, samples from three mice were pooled for a determination of the results. The tissues were minced with scissors and homogenized with a Kinematica Polytron homogenizer in 19 volumes of ice-cold 30 mM $Na^+$/HEPES buffer (pH 7.5). The homogenates were then centrifuged at 40,000 $\times$ *g* for 20 min and the resulting pellet was suspended in ice-cold buffer for the binding assay. In the *ex vivo* experiments, it is possible that oxybutynin and solifenacin can dissociate from the receptor sites during tissue preparation (homogenization and suspension). Yamada *et al*. (1980) have shown that such dissociation is extremely slow at 4°C, hence all preparation was conducted at this temperature. Protein concentrations were measured according to the method of Lowry *et al*. (1951). Mouse plasma was isolated from blood by centrifugation and stored at −80°C until analysis.

### *Muscarinic receptor binding assay*

The binding assay for muscarinic receptors was performed using [*N*-methyl-$^3$H]-scopolamine methyl chloride ([$^3$H]-NMS) as previously described (Ehlert & Tran, 1990; Oki *et al*., 2004). The mouse tissue homogenates (60–860 $\mu$g protein) were incubated with different concentrations (0.06–1.0 nM) of [$^3$H]-NMS in 30 mM $Na^+$/HEPES buffer (pH 7.5) for 60 min at 25°C. The reaction was terminated by rapid filtration (Cell Harvester, Brandel Co., Gaithersburg, MD, U.S.A.) through Whatman GF/B glass fibre filters, and the filters were then rinsed three times with 3 ml of ice-cold buffer. Tissue-bound radioactivity was extracted from the filters by overnight immersion in scintillation fluid (2 1 toluene, 1 1 Triton X-100, 15 g 2,5-diphenyloxazole, 0.3 g 1,4-bis[2-(5-phenyloxazolyl)]benzene), and radioactivity was determined with a liquid scintillation counter. Specific [$^3$H]-NMS binding was determined as the difference between counts in the absence and presence of 1 $\mu$M atropine. All assays were performed in duplicate.

### *Measurement of plasma concentrations*

Concentrations of oxybutynin and its active metabolite (*N*-desethyl-oxybutynin: DEOB) in mouse plasma were determined by gas chromatography and mass spectrometry (GC/MS) as described previously (Oki *et al*., 2004). A plasma sample (0.1–0.5 ml) was mixed with internal standard ([$^2H_{13}$]-oxybutynin · HCl and [$^2H_{13}$]-DEOB · HCl) and, after alkalinization with 0.5 ml of 0.5 M carbonate buffer (pH 9.5), extracted with 6 ml of *n*-hexane. After centrifugation at 1500 $\times$ *g* for 5 min, the supernatant was evaporated to dryness under reduced pressure. The residue was dissolved in 100 $\mu$l of $CH_3CN$, and 0.5–1 $\mu$l was injected into a GC/MS system consisting of a 5890 Series II gas chromatograph, a 5792 Series mass selective detector, a 7673 GC/SFC injector, a VECTRA 486/66U computer and a LaserJet 4 printer (Hewlett Packard Co.). Chromatographic separation was carried out using a 15 m $\times$ 0.25 mm i.d. $\times$ 0.25 $\mu$m film UA$^+$-1 HT (Frontier Lab Ltd.). The carrier gas was helium at a flow rate of 1.0 ml min$^{-1}$. Oven temperature was held at 150°C for 1 min, then programmed from 150 to 220°C at 20°C min$^{-1}$ for the first ramp, from 220 to 260°C at 10°C min$^{-1}$ for the second ramp, and from 260 to 300°C at 30°C min$^{-1}$ for the third ramp. It was held at 300°C for 2 min and then returned to the initial starting temperature of 150°C. The injection temperature was 200°C. Fragmentation was accomplished by electron impact at 70 eV ionizing voltage and 300 $\mu$A ionizing current. Selected ion monitoring was performed at *m*/*z* 342 (oxybutynin), *m*/*z* 355 (internal standard: [$^2H_{13}$]-oxybutynin), *m*/*z* 189 (DEOB) and *m*/*z* 200 (internal standard: [$^2H_{13}$]-DEOB). The limits of detection of oxybutynin and DEOB in plasma were 1.40 and 3.04 nM, respectively.

The concentration of solifenacin in mouse plasma was determined by a validated high-performance liquid chromatographic (HPLC) method. Briefly, solifenacin was extracted from mouse plasma by a two-step liquid–liquid extraction and analysed by semi-micro HPLC with UV detection. The lower limit of detection was 20.8 nM.

### *Measurement of salivary secretion*

Mice were anaesthetized with pentobarbitone (161 $\mu$mol kg$^{-1}$, intraperitoneally (i.p.)). Any saliva remaining in the oral cavity was removed with a cotton ball before measuring the total saliva collected in the cavity for a 10-min period. The saliva was absorbed onto three to five cotton balls for 10 min and the balls were weighed on an electric balance immediately after the collection period to prevent moisture loss. To examine the effects of oral administration of oxybutynin and solifenacin on pilocarpine-induced salivary secretion, pilocarpine (4.09 $\mu$mol kg$^{-1}$, dissolved in physiological saline) was administered (intravenous, i.v.) 0.5, 2, 6, 12 and 24 h after oral administration of the drugs, and saliva was collected for 10 min. The effect of oral administration of oxybutynin and solifenacin on total salivary secretion induced by cumulative doses (0.41–40.9 $\mu$mol kg$^{-1}$, i.v.) of pilocarpine given at 5-min intervals was also measured.

### *Data analysis*

Analysis of [$^3$H]-NMS binding data was performed as described previously (Yamada *et al*., 1980). The apparent dissociation constant ($K_D$) and maximal number of binding sites ($B_{max}$) for [$^3$H]-NMS were obtained by Rosenthal analysis of the saturation data. The ability of muscarinic receptor antagonists to inhibit specific [$^3$H]-NMS binding was determined from the $IC_{50}$ values, namely the molar concentration of unlabelled drug needed to displace 50% of the specific binding of [$^3$H]-NMS (determined by log probit analysis). The inhibition constant, $K_i$, was calculated from the equation $K_i = IC_{50}/(1 + L/K_D)$, where $L$ is the concentration of [$^3$H]-NMS (0.13 nM), and p$K_i$ ($-\log K_i$) values determined. Also, Hill slopes for the inhibition by muscarinic receptor antagonists were calculated.

For the analysis of salivary secretion data, p$K_B$ values were calculated using the equation p$K_B = \log (CR-1) - \log$ [antagonist], where CR is the ratio of $ED_{50}$ values (the dose producing 50% of the maximum response) of the agonist with and without antagonist (van Rossum *et al*., 1963).

Statistical analysis of the data was performed by one-way analysis of variance (ANOVA) followed by Dunnett's test for multiple comparisons. A value of $P<0.05$ was considered significant.

### *Materials*

[$^3$H]-NMS (3.03 TBq mmol$^{-1}$) was purchased from Perkin-Elmer Life Sciences, Inc. (Boston, MA, U.S.A.). Solifenacin succinate (YM905) was donated by Yamanouchi Pharmaceutical Co. Ltd (Tsukuba, Japan). Oxybutynin hydrochloride and its active metabolite (DEOB) were donated by Meiji Milk Products Co. Ltd (Odawara, Japan). All other chemicals were purchased from commercial sources.

## Results

### In vitro *effects of oxybutynin, DEOB and solifenacin on muscarinic receptors in mouse tissues*

Oxybutynin (1–1000 nM) and DEOB (0.3–100 nM) inhibited specific [$^3$H]-NMS binding in the bladder, submaxillary gland and heart in a concentration-dependent manner *in vitro*. Respective p$K_i$ values for oxybutynin in these tissues were 7.80, 8.22 and 7.39, while those for DEOB were 8.30, 9.00 and 7.80 (Table 1). The inhibitory potencies for DEOB in the bladder, submaxillary gland and heart were thus significantly ($P<0.01$) higher (3.1, 6.0 and 2.6 times, respectively) than those obtained for oxybutynin.

Solifenacin (3–1000 nM) inhibited specific [$^3$H]-NMS binding in the bladder, submaxillary gland and heart, and the associated p$K_i$ values were 7.38, 7.89 and 7.00, respectively (Table 1). The inhibitory effect of solifenacin was significantly lower (1/3.2) in the submaxillary gland and higher (2.4 times) in the heart than in the bladder. Further, the inhibitory effects of solifenacin in the bladder, submaxillary gland and heart were significantly lower (1/2.6, 1/2.1 and 1/2.4 respectively) than those of oxybutynin.

The Hill slopes for oxybutynin, DEOB and solifenacin in these tissues were close to unity except that for DEOB in the submaxillary gland which was 0.78.

### *Effects of oral administration of oxybutynin and solifenacin on muscarinic receptors in mouse tissues*

At 0.5 and/or 2 h after oral administration of oxybutynin (76.1 $\mu$mol kg$^{-1}$), there was a significant increase in the $K_D$ value for specific [$^3$H]-NMS binding in the bladder, prostate, submaxillary gland, heart, lung and colon compared with the corresponding control values (Table 2). The increases were maximal after 0.5 h being 54.5, 220, 668, 91.7, 391 and 269%, respectively, while those at 2 h were 44.3, 218, 215, 16.9, 115 and 109%, respectively. The increase at 0.5 h was least in the bladder and most in the submaxillary gland. The $K_D$ in each tissue was not significantly different from the control value at 6, 12 and 24 h. Oxybutynin had no significant effect on $B_{max}$ values for specific [$^3$H]-NMS binding in any of the tissues.

Oral administration of solifenacin (62.4 $\mu$mol kg$^{-1}$) significantly increased the $K_D$ value for specific [$^3$H]-NMS binding in all the tissues when compared with the corresponding control values (Table 3). These effects were significant in the heart (24.0 and 25.1%) and lung (244 and 182%) at 0.5 and 2 h, in the bladder (18.2–29.0%) and submaxillary gland (106–363%) at 0.5 to 6 h, and in the prostate (72.7–120%) and colon (83.6–85.6%) at 0.5 to 12 h. In addition, the maximum effect in each

**Table 1** p$K_i$ values for *in vitro* inhibition by oxybutynin, DEOB and solifenacin of specific [$^3$H]-NMS binding in the bladder, submaxillary gland and heart of mice

| *Drug* | *p*K$_i$ *value* | | |
|---|---|---|---|
| | *Bladder* | *Submaxillary gland* | *Heart* |
| Oxybutynin | 7.80 ± 0.03$^{††}$ | 8.22 ± 0.03**$^{††}$ | 7.39 ± 0.06**$^{††}$ |
| | (0.98 ± 0.03) | (0.87 ± 0.07) | (1.03 ± 0.05) |
| DEOB | 8.30 ± 0.08$^{††}$ | 9.00 ± 0.05**$^{††}$ | 7.80 ± 0.02**$^{††}$ |
| | (0.84 ± 0.09) | (0.78 ± 0.04) | (1.08 ± 0.03) |
| Solifenacin | 7.38 ± 0.03 | 7.89 ± 0.03** | 7.00 ± 0.03** |
| | (0.95 ± 0.03) | (0.90 ± 0.02) | (1.00 ± 0.06) |

Values are mean ± s.e.m. of three or four mice. Values in parentheses represent Hill slopes. Asterisks show a significant difference from values in the bladder, **$P<0.01$. Daggers show a significant difference from the values with solifenacin, $^{††}P<0.01$.

**Table 2** $K_D$ and $B_{max}$ for specific [$^3$H]-NMS binding in the bladder, prostate, submaxillary gland, heart, lung and colon of mice 0.5–24 h after oral administration of oxybutynin (76.1 $\mu$mol kg$^{-1}$)

| *Organ* | *Time after oral oxybutynin* (h) | $K_D$ (pM) | $B_{max}$ (fmol mg protein$^{-1}$) |
|---|---|---|---|
| Bladder | Control | 176±4 | 152±8 |
| | 0.5 | 272±24 (1.55)** | 120±12 |
| | 2 | 254±27 (1.44)** | 141±8 |
| | 6 | 221±5 | 138±14 |
| | 12 | 190±10 | 139±14 |
| | 24 | 178±8 | 135±5 |
| Prostate | Control | 124±3 | 160±17 |
| | 0.5 | 397±53 (3.20)*** | 120±25 |
| | 2 | 394±78 (3.18)*** | 159±30 |
| | 6 | 246±33 | 137±14 |
| | 12 | 182±21 | 151±24 |
| | 24 | 129±5 | 166±20 |
| Submaxillary gland | Control | 111±2 | 141±8 |
| | 0.5 | 852±149 (7.68)*** | 140±6 |
| | 2 | 350±17 (3.15)* | 119±10 |
| | 6 | 181±14 | 166±17 |
| | 12 | 138±4 | 146±5 |
| | 24 | 113±1 | 154±8 |
| Heart | Control | 278±12 | 43.1±1.1 |
| | 0.5 | 533±67 (1.92)*** | 46.2±0.8 |
| | 2 | 325±13 | 47.9±2.8 |
| | 6 | 315±8 | 47.8±1.1 |
| | 12 | 276±10 | 40.4±1.5 |
| | 24 | 257±9 | 44.8±3.2 |
| Lung | Control | 185±10 | 84.2±4.9 |
| | 0.5 | 909±153 (4.91)*** | 84.1±15.3 |
| | 2 | 398±29 (2.15)* | 89.2±5.3 |
| | 6 | 273±13 | 81.0±4.6 |
| | 12 | 202±6 | 102±10 |
| | 24 | 186±5 | 110±5 |
| Colon | Control | 170±3 | 150±7 |
| | 0.5 | 628±100 (3.69)*** | 165±18 |
| | 2 | 355±25 (2.09)* | 143±9 |
| | 6 | 321±35 | 162±8 |
| | 12 | 234±19 | 169±6 |
| | 24 | 182±8 | 160±5 |

Rosenthal analysis was performed with [$^3$H]-NMS (0.06–1.0 nM) binding in mouse tissues after oral oxybutynin (76.1 $\mu$mol kg$^{-1}$). Values are mean±s.e.m. of three to five mice. Values in parentheses represent the fold-increase in $K_D$ values relative to controls. Asterisks show a significant difference from the control values, *$P<0.05$, **$P<0.01$, ***$P<0.001$.

**Table 3** $K_D$ and $B_{max}$ for specific [$^3$H]-NMS binding in the bladder, prostate, submaxillary gland, heart, lung and colon of mice 0.5–24 h after oral administration of solifenacin (62.4 $\mu$mol kg$^{-1}$)

| *Organ* | *Time after oral solifenacin* (h) | $K_D$ (pM) | $B_{max}$ (fmol mg protein$^{-1}$) |
|---|---|---|---|
| Bladder | Control | 176±6 | 169±22 |
| | 0.5 | 208±9 (1.18)* | 156±25 |
| | 2 | 227±6 (1.29)*** | 139±17 |
| | 6 | 208±11(1.18)* | 126±10 |
| | 12 | 188±7 | 123±26 |
| | 24 | 182±4 | 123±18 |
| Prostate | Control | 110±4 | 170±23 |
| | 0.5 | 217±34 (1.97)*** | 221±33 |
| | 2 | 242±8 (2.20)*** | 191±23 |
| | 6 | 203±3 (1.85)*** | 203±8 |
| | 12 | 190±14 (1.73)*** | 219±24 |
| | 24 | 129±2 | 186±9 |
| Submaxillary gland | Control | 96.7±3.1 | 122±9 |
| | 0.5 | 233±35 (2.41)*** | 116±13 |
| | 2 | 448±36 (4.63)*** | 116±8 |
| | 6 | 199±12 (2.06)** | 115±14 |
| | 12 | 145±16 | 109±4 |
| | 24 | 96.6±0.8 | 112±8 |
| Heart | Control | 283±12 | 35.4±0.9 |
| | 0.5 | 351±11 (1.24)** | 43.2±4.6 |
| | 2 | 354±9 (1.25)** | 39.9±4.2 |
| | 6 | 305±12 | 46.1±3.7 |
| | 12 | 301±15 | 44.1±3.1 |
| | 24 | 288±11 | 44.2±2.8 |
| Lung | Control | 177±7 | 80.4±3.1 |
| | 0.5 | 608±86 (3.44)*** | 80.9±6.9 |
| | 2 | 500±41 (2.82)*** | 80.8±15.0 |
| | 6 | 274±7 | 69.1±4.3 |
| | 12 | 225±24 | 66.5±3.8 |
| | 24 | 186±6 | 60.5±0.8 |
| Colon | Control | 146±12 | 131±14 |
| | 0.5 | 271±34 (1.86)*** | 121±9 |
| | 2 | 268±12 (1.84)*** | 150±18 |
| | 6 | 270±10 (1.85)*** | 123±8 |
| | 12 | 271±10 (1.86)*** | 139±10 |
| | 24 | 162±7 | 141±4 |

Rosenthal analysis was performed with [$^3$H]-NMS (0.06–1.0 nM) binding in mouse tissues after oral solifenacin (62.4 $\mu$mol kg$^{-1}$). Values are mean±s.e.m. of three to seven mice. Values in parentheses represent the fold-increase in $K_D$ values relative to controls. Asterisks show a significant difference from the control values, *$P<0.05$, **$P<0.01$, ***$P<0.001$.

tissue was seen at 0.5, 2 h or both. Further, oral administration of solifenacin at an approximately three-fold higher dose (208 $\mu$mol kg$^{-1}$) further increased the $K_D$ values for [$^3$H]-NMS binding in each tissue (Table 4), with increases being significant in the heart (56.5 and 47.0%, respectively) at 0.5 and 2 h, and in the bladder (22.2–49.4%), prostate (166–320%), submaxillary gland (234–613%) and colon (116–131%) at 0.5 to 12 h. Solifenacin had no significant effect on the $B_{max}$ value for any of the tissues, except in the heart where a significant (38.1%) increase occurred at 2 h (at the dose of 208 $\mu$mol kg$^{-1}$).

### *Plasma levels of oxybutynin, DEOB and solifenacin*

Figure 1 shows the plasma concentrations of oxybutynin, DEOB and solifenacin over time after oral administration of oxybutynin and solifenacin. The concentrations of oxybutynin and DEOB reached maximum levels (oxybutynin, 91.5±34.3 nM; DEOB, 75.8±20.6 nM; $n=7$–9) 0.5 h after administration of oxybutynin (76.1 $\mu$mol kg$^{-1}$) and then they rapidly declined (Figure 1a). Respective plasma concentrations of oxybutynin at 2 and 6 h were 16.3±2.7 and 3.15±1.54 nM, while those of DEOB were 28.8±5.3 and 2.18±2.18 nM.

**Table 4** $K_D$ and $B_{max}$ for specific [$^3$H]-NMS binding in the bladder, prostate, submaxillary gland, heart, lung and colon of mice 0.5–24 h after oral administration of solifenacin (208 $\mu$mol kg$^{-1}$)

| *Organ* | *Time after oral solifenacin* (h) | $K_D$ (pM) | $B_{max}$ (fmol mg protein$^{-1}$) |
|---|---|---|---|
| Bladder | Control | 176±6 | 169±22 |
| | 0.5 | 240±1 (1.36)*** | 138±21 |
| | 2 | 263±15 (1.49)*** | 156±11 |
| | 6 | 243±16 (1.38)*** | 141±26 |
| | 12 | 215±9 (1.22)* | 139±15 |
| | 24 | 175±8 | 124±19 |
| Prostate | Control | 110±4 | 170±23 |
| | 0.5 | 293±28 (2.66)*** | 215±33 |
| | 2 | 462±37 (4.20)*** | 168±15 |
| | 6 | 396±37 (3.60)*** | 170±34 |
| | 12 | 340±11 (3.09)*** | 234±34 |
| | 24 | 142±11 | 197±18 |
| Submaxillary gland | Control | 96.7±3.1 | 122±9 |
| | 0.5 | 455±27 (4.71)*** | 129±9 |
| | 2 | 689±79 (7.13)*** | 97.2±2.5 |
| | 6 | 469±18 (4.85)*** | 120±16 |
| | 12 | 323±15 (3.34)*** | 111±11 |
| | 24 | 110±4 | 136±13 |
| Heart | Control | 283±12 | 35.4±0.9 |
| | 0.5 | 443±22 (1.57)*** | 42.2±3.0 |
| | 2 | 416±21 (1.47)*** | 48.9±4.1* |
| | 6 | 321±25 | 40.7±6.7 |
| | 12 | 319±7 | 46.6±2.3 |
| | 24 | 278±13 | 43.4±2.6 |
| Lung | Control | 177±7 | 80.4±3.1 |
| | 0.5 | 468±278 | 67.8±8.3 |
| | 2 | 806±54 (4.55)** | 60.0±5.9 |
| | 6 | 488±25 | 67.7±0.6 |
| | 12 | 408±15 | 78.4±11.5 |
| | 24 | 196±7 | 62.1±2.3 |
| Colon | Control | 146±12 | 131±14 |
| | 0.5 | 334±38 (2.29)*** | 126±8 |
| | 2 | 315±8 (2.16)*** | 167±13 |
| | 6 | 337±18 (2.31)*** | 126±11 |
| | 12 | 320±25 (2.19)*** | 147±13 |
| | 24 | 172±16 | 156±24 |

Rosenthal analysis was performed with [$^3$H]-NMS (0.06–1.0 nM) binding in mouse tissues after oral solifenacin (208 $\mu$mol kg$^{-1}$). Values are mean±s.e.m. of three to seven mice. Values in parentheses represent the fold-increase in $K_D$ values relative to controls. Asterisks show a significant difference from the control values, *$P<0.05$, **$P<0.01$, ***$P<0.001$.

Solifenacin at doses of 62.4 and 208 $\mu$mol kg$^{-1}$ produced dose-dependent increases in its plasma concentration. Maximum levels were reached at 0.5 h (886±176 nM; $n=6$) and 2 h (1381±201 nM; $n=6$), respectively, followed by a gradual decline (Figure 1b) to 546±84, 178±20, 127±29 and 38.5 nM at 2, 6, 12 and 24 h, respectively, and to 804±233, 283±40 and 38.7±6.0 nM at 6, 12 and 24 h, respectively.

## *Salivary secretion*

Figure 2 shows the effect of oral administration of oxybutynin and solifenacin on pilocarpine-induced salivary secretion in mice. Salivary secretion induced by an i.v. injection of pilocarpine in control mice showed good reproducibility, with similar amounts of saliva being secreted during the 10-min collection period at 0.5, 2, 6, 12 and 24 h after stimulation (293±18, 263±14, 228±9, 234±15 and 252±9 mg, respectively ($n=12$)). Pilocarpine-induced secretion was significantly decreased at 0.5 and 2 h after oral administration of oxybutynin (76.1 $\mu$mol kg$^{-1}$), compared with the control value; the response to pilocarpine was almost abolished at 0.5 h but secretion recovered to the control level at 6 h.

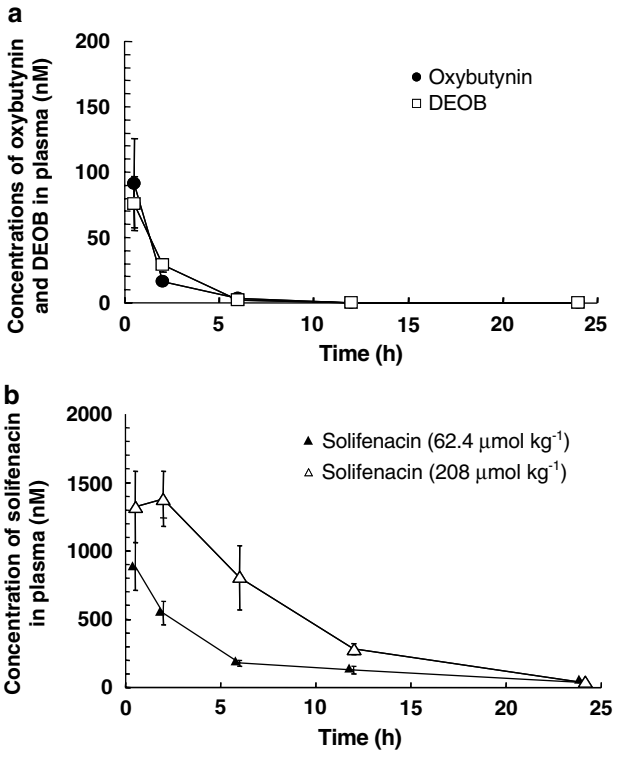


**Figure 1** Plasma concentrations of oxybutynin, DEOB and solifenacin over time after oral administration of oxybutynin (a) and solifenacin (b) in mice. Mice received oxybutynin (76.1 $\mu$mol kg$^{-1}$) or solifenacin (62.4, 208 $\mu$mol kg$^{-1}$) orally, and were then killed. Blood samples were taken from the descending aorta of each mouse. Each point represents the mean±s.e.m. of one to nine mice.

Similarly, pilocarpine-induced salivary secretion was markedly reduced but not abolished at 0.5–6 h after oral administration of solifenacin (62.4 and 208 $\mu$mol kg$^{-1}$), as shown by residual saliva (62.4 $\mu$mol kg$^{-1}$: 0.5 h, 66.0±20.4 mg; 2 h, 54.6±23.3 mg; 6 h, 75.2±19.6 mg; and 208 $\mu$mol kg$^{-1}$: 0.5 h, 36.6±20.8 mg; 2 h, 11.8±2.3 mg; 6 h, 44.2±15.6 mg). There was little difference in the secretion responses between the control and solifenacin treatment groups at 12 and 24 h.

As shown in Figure 3, cumulative doses of pilocarpine (0.41–40.9 $\mu$mol kg$^{-1}$, i.v.) induced dose-dependent salivary secretion. To determine the effects of oxybutynin and solifenacin on these dose–response curves, secretion was measured at the time of maximum inhibition of pilocarpine-induced salivation, that is 0.5 h for oxybutynin and 2 h for solifenacin (Figure 2). Both oxybutynin (76.1 $\mu$mol kg$^{-1}$) and solifenacin (62.4, 208 $\mu$mol kg$^{-1}$) caused a rightward shift in the dose–response curve for pilocarpine-induced salivation, but the inhibitory effect of solifenacin, 62.4 $\mu$mol kg$^{-1}$, was significantly weaker than that of oxybutynin (76.1 $\mu$mol kg$^{-1}$).

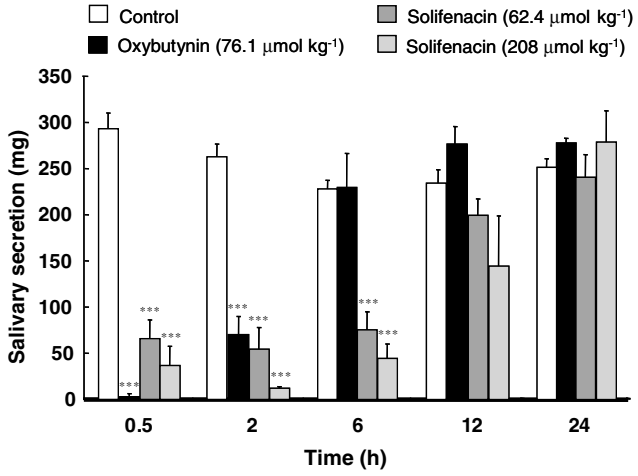


**Figure 2** Effects of oral administration of oxybutynin and solifenacin on pilocarpine-induced salivary secretion in mice. Mice received oxybutynin (76.1 $\mu$mol kg$^{-1}$) or solifenacin (62.4, 208 $\mu$mol kg$^{-1}$) orally, and then the total saliva was collected for 10 min with absorbent cotton balls following pilocarpine stimulation (4.09 $\mu$mol kg$^{-1}$, i.v.). Each column represents the mean $\pm$ s.e.m. of 4–12 mice. Asterisks show a significant difference from control values, ***$P<0.001$.

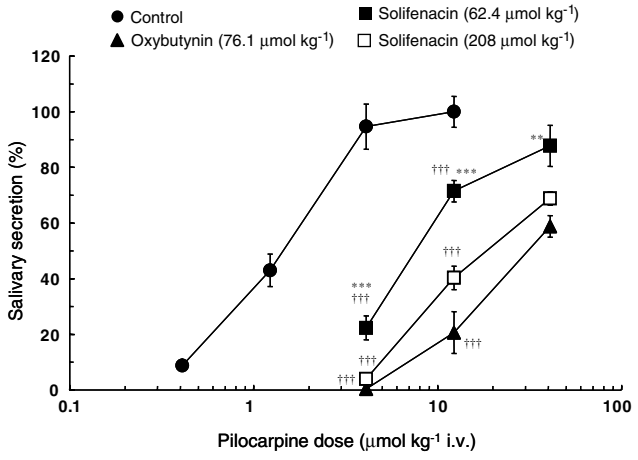


**Figure 3** Effects of oral administration of oxybutynin and solifenacin on the dose–response curve of pilocarpine-induced salivary secretion in mice. Mice received cumulative intravenous doses (0.41–40.9 $\mu$mol kg$^{-1}$) of pilocarpine at 5-min intervals from 0.5 h after oral administration of oxybutynin (76.1 $\mu$mol kg$^{-1}$) and 2 h after oral administration of solifenacin (62.4, 208 $\mu$mol kg$^{-1}$), and then their total saliva was collected. Each point represents the mean $\pm$ s.e.m. of four to nine mice. Daggers show a significant difference from each control value, $^{\dagger\dagger\dagger}P<0.001$. Asterisks show a significant difference from each value of oxybutynin, **$P<0.01$, ***$P<0.001$.

Thus, the p$K_B$ value for oxybutynin (5.53 $\pm$ 0.03) was significantly greater ($P<0.001$) than that for solifenacin (4.85 $\pm$ 0.05).

## Discussion

The usefulness of *ex vivo* and *in vivo* receptor binding assays in predicting the potency, organ selectivity and duration of action of drugs in relation to their pharmacokinetic and pharmacodynamic profiles is well documented (Beauchamp *et al*., 1995; Uchida *et al*., 1995; Ohkura *et al*., 1998; Yamada *et al*., 2001; 2002; 2003). In the present study, the *in vivo* muscarinic receptor binding of solifenacin was characterized and compared to that of oxybutynin by simultaneously measuring [$^3$H]-NMS binding in various tissues, including the bladder and submaxillary gland. Also their plasma concentrations and effects on salivary secretion after oral administration were compared. The doses of solifenacin given (2.1–208 $\mu$mol kg$^{-1}$) were similar to those shown to have an inhibitory effect in experimental models of bowel dysfunction *in vivo* (Kobayashi *et al*., 2001).

It is known that the heart and salivary gland contain predominantly $M_2$ and $M_3$ muscarinic subtypes, respectively (Giraldo *et al*., 1988; Caulfield, 1993), whereas the bladder contains both, with the $M_2$ dominating over the $M_3$ subtype (Wang *et al*., 1995). Furthermore, functional and binding studies have shown that, although $M_3$-receptors are the main contributors, $M_1$, $M_4$ and $M_5$ receptor subtypes may also have a role in muscarinic agonist-induced salivation in mice (Hammer *et al*., 1980; Yeomans *et al*., 2001; Takeuchi *et al*., 2002; Bymaster *et al*., 2003; Gautam *et al*., 2004; Nakamura *et al*., 2004). In the present *in vitro* experiments, both solifenacin and oxybutynin competed with [$^3$H]-NMS for binding sites in the bladder, submaxillary gland and heart of mice in a concentration-dependent manner. The p$K_i$ values indicated that the muscarinic receptor binding affinity of solifenacin in these tissues was 2–3 times weaker than that of oxybutynin. Ikeda *et al*. (2002) have previously found that the p$K_i$ values of solifenacin for $M_1$, $M_2$ and $M_3$ subtypes in binding assays were 7.6, 6.9 and 8.0, respectively. Thus, p$K_i$ values of this agent in the mouse heart and submaxillary gland (7.0 and 7.89; Table 1) agree well with the respective $M_2$ and $M_3$ values, while in the bladder the p$K_i$ (7.4) was intermediate between these two subtypes. A similar correlation in relative affinity between recombinant receptor subtypes and native tissues has been observed for oxybutynin (Ikeda *et al*., 2002).

After oral administration of solifenacin (62.4, 208 $\mu$mol kg$^{-1}$) and oxybutynin (76.1 $\mu$mol kg$^{-1}$), dose- and time-dependent increases in $K_D$ values for specific [$^3$H]-NMS binding were seen in the bladder, prostate, submaxillary gland, heart, colon and lung of mice, with little effect on $B_{max}$ values. Given that an increase in $K_D$ values for radioligands in drug-pretreated tissues in this type of assay usually indicates competition between the agent and radioligand for the same binding sites (Ohkura *et al*., 1998; Yamada *et al*., 2003), these data strongly suggest that orally administered solifenacin and oxybutynin undergo significant binding to muscarinic receptors in these tissues. Differences were seen between the two drugs in the time course of their effects on the $K_D$ values for [$^3$H]-NMS binding. With oxybutynin, the increase in $K_D$ in each tissue reached a maximum 0.5 h after oral administration, followed by a rapid decline. In contrast, the increase in $K_D$ with solifenacin in most tissues was greatest at 2 h and was maintained for up to 6 or 12 h, depending on dose; its effect was slower in onset and longer in duration compared to that of oxybutynin. This apparent difference between solifenacin and oxybutynin appears to depend largely on their rate of increase and disappearance in the plasma. The plasma concentration of oxybutynin reached a maximum 0.5 h after oral administration and then declined rapidly, whereas

that of solifenacin peaked at 0.5 or 2 h and then declined more slowly. Based on the intensity and duration of the increases in $K_D$ values, the muscarinic receptor binding activity of solifenacin is greatest in the submaxillary gland and lowest in the heart and persistent in the bladder, prostate, submaxillary gland and colon but transient in the heart and lung. Recent data with $M_1$–$M_5$ subtype knockout mice show that the $M_3$ subtype is expressed predominantly (70–80%) in the submaxillary gland and moderately in the prostate and bladder, whereas $M_2$ is the main subtype present in the bladder, heart, lung and colon (Oki *et al*., unpublished observation). The tissue selectivity of oral solifenacin may therefore reflect the muscarinic subtype selectivity shown in the *in vitro* assay (Ikeda *et al*., 2002, Table 1). Consequently, it is possible that the higher affinity of solifenacin for the $M_3$ compared to the $M_2$ subtype accounts for its persistent binding to muscarinic receptors in the mouse bladder, prostate and submaxillary gland and transient binding in $M_2$-predominant tissues. As mouse salivary gland has recently been shown to contain functional $M_1$, $M_4$ and $M_5$-receptor subtypes, the partial binding of solifenacin to these non-$M_3$-receptor subtypes cannot be ruled out. Moreover, it should be noted that NMS itself displays higher affinity for the $M_3$ than $M_2$ subtype (Waelbroeck *et al*., 1990). The persistent binding of solifenacin in the mouse colon remains to be clarified.

It is possible that solifenacin, similar to the long-lasting 1,4-dihydropyridine calcium channel antagonists, benidipine and amlodipine (Yamada *et al*., 2002) has slower receptor binding kinetics of association and dissociation. In our preliminary experiments *in vitro*, no significant difference was observed between solifenacin and oxybutynin in their association rates of muscarinic receptor binding in the submaxillary gland and heart. Solifenacin was more readily dissociated from muscarinic receptors in the submaxillary gland compared with the heart, while the dissociation rate of oxybutynin was similar in both tissues. These data do not appear to support the results obtained *ex vivo* after oral administration of these agents (Tables 2–4). However, kinetic data obtained from *in vitro* studies may not necessarily reflect receptor binding kinetics under *in vivo* conditions, which could be greatly influenced by various pharmacokinetic factors, regional blood flow and intrinsic cholinergic neuronal activity in each tissue. A comparative kinetic analysis of drug concentration and muscarinic receptor binding in tissues of mice administered radiolabelled forms of solifenacin and oxybutynin might clarify any differences in the *in vivo* binding characteristics of these agents and also between tissues.

Recently, in contrast to our results, Nelson *et al*. (2004) found that oxybutynin selectively inhibits carbachol-stimulated phosphoinositide responses in slices of bladder from the guinea-pig when compared to the submandibular gland. Our finding, in mice, that oxybutynin binds more to receptors in the submaxillary gland than the bladder may be due to various factors present in *in vivo* experiments that could affect the concentration of drug reaching a tissue. These include a rapid rise of plasma drug concentration occurring after oral oxybutynin, the amount and rate of drug reaching each tissue depending on the organ blood flow and the formation of the active metabolite (DEOB). In fact, we have recently shown that i.v. injected DEOB, similar to oxybutynin, binds more extensively to muscarinic receptors in the submaxillary gland than in the bladder of rats (Oki *et al*., 2005).

It is possible that in our study the doses of solifenacin used exert anticholinergic effects in the bladder. In our preliminary experiments 93.2% of solifenacin bound to plasma protein and the free fractions present in plasma were estimated to be 60.2 nM (0.5 h), 37.1 nM (2 h) and 12.1 nM (6 h) at 62.4 $\mu$mol kg$^{-1}$, and 93.9 nM (2 h), 54.7 nM (6 h) and 19.2 nM (12 h) at 208 $\mu$mol kg$^{-1}$. As the *in vitro* $K_i$ value of this agent in displacing bladder [$^3$H]-NMS binding is 41.9 nM, it is possible that solifenacin at the oral doses used elicits significant blockade of muscarinic receptors in this tissue. In fact, we have shown that solifenacin at oral doses of 2.1–62.4 $\mu$mol kg$^{-1}$ significantly attenuates the carbachol-induced increase in mouse intravesical pressure in a dose-dependent manner (Sato *et al*., unpublished observations).

In *in situ* experiments in anaesthetized rats, Ikeda *et al*. (2002) have shown that intravenous solifenacin is 2 and 6.5 times weaker than oxybutynin at inhibiting carbachol-induced bladder contractions and salivary secretion, respectively, indicating the selectivity of solifenacin for the bladder. Similarly, Ohtake *et al*. (2004) found that solifenacin had a greater selectivity for the urinary bladder compared to the salivary gland in rats. In the present study, pilocarpine-induced salivary secretion in mice was markedly reduced 0.5–6 h after oral solifenacin (62.4 and 208 $\mu$mol kg$^{-1}$), but was completely abolished 0.5 h after oral administration of oxybutynin (76.1 $\mu$mol kg$^{-1}$). The rapid cessation of salivary secretion induced by oral oxybutynin did not occur in mice receiving oral solifenacin and the p$K_B$ values showed that solifenacin was significantly (5 times) weaker than oxybutynin, at inhibiting pilocarpine-induced salivation. Thus, the intensity and time-course of the inhibitory effects of solifenacin and oxybutynin on salivary secretion after oral administration appear to accord well with those for muscarinic receptor binding in the submaxillary gland.

After oral administration of oxybutynin, similar concentrations of DEOB as those for oxybutynin were detected in the plasma of mice. The *in vitro* receptor binding affinity of DEOB in mouse tissues was 3–6 times higher than that of oxybutynin, with the highest affinity (6 times that of oxybutynin) occurring in the submaxillary gland. In man, Waldeck *et al*. (1997) showed a higher affinity of DEOB for muscarinic receptors in the parotid gland relative to the bladder. These data suggest that DEOB contributes more significantly to the blockade of muscarinic receptors in exocrine gland receptors than oxybutynin under *in vivo* conditions.

In conclusion, the results from the present study, show that oral solifenacin significantly binds to muscarinic receptors in various tissues of mice, including the bladder, and that this binding is persistent in tissues expressing the $M_3$ subtype. Further, the inhibitory effect of solifenacin on pilocarpine-evoked salivary secretion was significantly weaker but more persistent than that of oxybutynin.

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