In Vitro and *in Vivo* Trans-Esterification of 1-[2(*R*)-(2-Amino-2-Methylpropionylamino)-3-(1H-Indol-3-yl)Propionyl]-3(*S*)-Benzyl-Piperidine-3-Carboxylic Acid Ethyl Ester and the Effects of Ethanol on Its Pharmacokinetics in Rats

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Received August 15, 2004; accepted March 2, 2004

Purpose. To investigate the *in vitro* trans-esterification of 1-[2(*R*)-(2-amino-2-methylpropionylamino)-3-(1H-indol-3-yl)propionyl]-3(*S*)-benzyl-piperidine-3-carboxylic acid ethyl ester (compound A) and to determine the effects of ethanol on its *in vivo* pharmacokinetics in male Sprague-Dawley rats.

Methods. The effects of deuterated [d_5]ethanol on the hydrolysis and trans-esterification of compound A in rat plasma and rat liver microsomes in the presence or absence of bis(*p*-nitrophenyl) phosphate (BNPP), a carboxylesterase inhibitor, were investigated. Following an oral pretreatment with deuterated ethanol in conjunction with an intravenous dose of compound A to rats, the pharmacokinetics of compound A and deuterated compound A were evaluated.

Results. It was observed that the amount of deuterated compound A generated increased with increasing amounts of deuterated ethanol in incubates, whereas the amount of hydrolyzed product (compound B) decreased. BNPP inhibited both the hydrolysis and the trans-esterification of compound A. Furthermore, the pharmacokinetics of compound A in rats receiving ethanol was altered, such that the plasma clearance decreased by 1.5-fold and the elimination rate constant decreased by 2-fold. Deuterated compound A was determined, confirming that trans-esterification proceeded *in vivo*; approximately one third of the intravenous dose of compound A underwent trans-esterification.

Conclusions. In the presence of ethanol, compound A underwent trans-esterification catalyzed by carboxylesterases. Ethanol pretreatment resulted in a decrease in the *in vivo* clearance of compound A mainly due to trans-esterification with ethanol.

KEY WORDS: ester drugs; ethanol; growth hormone secretagogue; pharmacokinetics; trans-esterification.

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ABBREVIATIONS: AUC, area under the plasma concentration vs. time curve; AUMC, first moment of the plasma concentration vs. time curve; BNPP, bis(*p*-nitrophenyl) phosphate; CL_p, plasma clearance; [d_5]ethanol, deuterated ethanol; GHS, growth hormone secretagogue; k_{el}, elimination rate constant; V_d, apparent volume of distribution.

INTRODUCTION

The compound, 1-[2(*R*)-(2-amino-2-methylpropionyl-amino)-3-(1H-indol-3-yl)propionyl]-3-benzylpiperidine-3(*S*)-carboxylic acid ethyl ester (compound A; Fig. 1) is a potent and short-acting growth hormone secretagogue (GHS) (1). *In vivo*, this compound is rapidly converted to its acid form, compound B, which is pharmacologically less active (Fig. 1). Ester-containing drugs or prodrugs may undergo trans-esterification when co-administered with ethanol and thereby cause changes in the pharmacokinetics of the drug. For example, trans-esterification was observed when cocaine, a methyl ester, was co-administered with ethanol, resulting in the formation of the ethyl ester of cocaine (2–5) and also decreasing the clearance of cocaine in the conscious dog (6). However, there is a dearth of published information about the effects of ethanol on the pharmacokinetics of other ester drugs or ester prodrugs. In the current study, the *in vitro* trans-esterification of compound A was investigated together with the effects of ethanol on the *in vivo* pharmacokinetics of compound A in rats.

MATERIALS AND METHODS

Materials

1-[2(*R*)-(2-amino-2-methylpropionylamino)-3-(1H-indol-3-yl)propionyl]-3(*S*)-benzyl-piperidine-3-carboxylic acid ethyl ester (compound A) and its carboxylic acid analog (compound B) were synthesized at Merck Research Laboratories (Rahway, NJ, USA) (1). Deuterated ethanol ([d_5]ethanol) and bis(*p*-nitrophenyl) phosphate (BNPP) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals and reagents were of the highest grade commercially available.

Incubation of Compound A with Rat Plasma or Rat Liver Microsomes in the Presence of [d_5]Ethanol and BNPP

Following deep CO_2 anesthesia, rat blood was obtained by cardiac puncture and collected into heparinized tubes. The blood was spun in a centrifuge at 3000 rpm for 10 min at 4°C to separate the plasma, which was kept on ice until use. Rat liver microsomes were prepared and pooled from four rats (7). Ten microliters of 50 μM aqueous solution of compound A and 10 μL of 0, 0.5, 1, 5, or 25% aqueous [d_5]ethanol were incubated with either 80 μl of rat plasma at 37°C for 5 min or with 30 μl of 0.1 M phosphate buffer (pH 7.4) plus 50 μl of male rat liver microsomes (4 mg protein/ml) at 37°C for 30 min. The reaction was halted by the addition of 0.3 ml acetonitrile, and the tubes were kept on ice for 1 h, followed by centrifugation at 3000 rpm for 10 min. In experiments involving BNPP, the incubates contained 2.5% (v/v) aqueous [d_5]ethanol and 5 μl of 0.2 M BNPP (in acetonitrile/water, 1:1, v/v).

In vivo Pharmacokinetic Studies

Adult, male, Sprague-Dawley rats (275–300 g) were purchased from Charles River Laboratories (Wilmington, MA, USA) and were housed in the Merck Laboratory Animal Resources facilities under the supervision of certified veterinarians. All animal experiments were approved by Merck's Institutional Animal Care and Use Committee. Animals were

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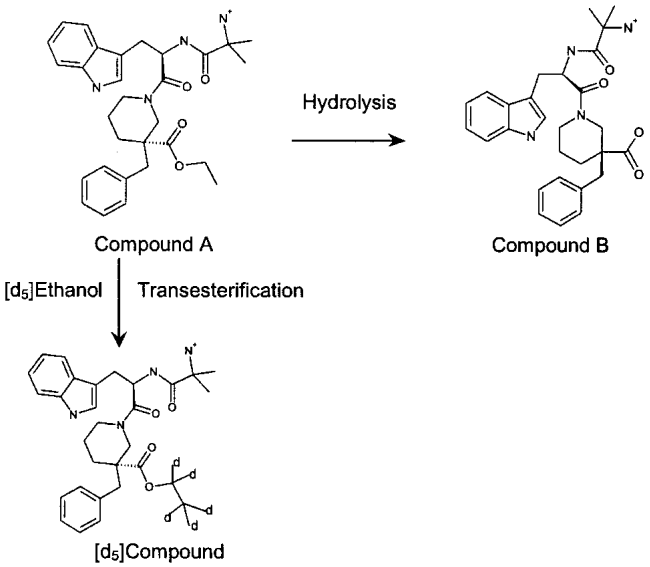

Fig. 1. Scheme for the hydrolysis and trans-esterification of compound A.

housed on hardwood bedding in plastic cages, with water and commercial rat diet supplied *ad libitum*. The animal facilities were maintained between 20°C and 23°C with a 12 h light/dark photoperiod. After a 1- to 2-week acclimation period, the animals were fasted overnight prior to dosing. Food was returned 4 h after dosing, and water was available to the animals throughout the experiment. Rats were randomly assigned to one of the three groups with six animals in the first two groups and four animals in the third group. The first, second, and third group of animals received an oral gavage of water, aqueous ethanol (ethanol/water, 1:1, v/v), or aqueous [d_5]ethanol (deuterated ethanol/water, 1:1, v/v) at 3 g/kg, respectively, 15 min prior to intravenous administration of compound A. All 16 animals received a bolus intravenous dose of compound A (5 mg/kg) *via* a cannula previously implanted into the femoral vein. Blood samples (350 μl) were collected into heparinized tubes, *via* a previously cannulated femoral artery, at 2.5, 5, 15, 30 min, and 1, 2, 3, 4, and 6 h post-dosing. To prevent the *ex vivo* ester hydrolysis of compound A, blood samples were placed on ice, and 30 μl of 0.5 M BNPP (in acetonitrile/water, 1:1, v/v) was added immediately to each tube (the hydrolysis of compound A in rat plasma was completely inhibited under these conditions). Blood samples were spun in a centrifuge at 3000 rpm for 10 min at 4°C to separate the plasma. Subsequently, 200 μl of each plasma sample was quenched with 1.25 ml ice-cold methanol, followed by centrifugation at 3000 rpm for 10 min. The supernatant was removed and retained. The pellet was re-extracted with 1.25 ml methanol and, after centrifugation, the supernatant was removed and combined with that from the first centrifugation. The combined supernatants were evaporated at room temperature under a stream of dry N_2, and the residue was reconstituted in 200 μl acetonitrile/water (9:1, v/v).

LC-MS/MS Assay

Quantitative analyses of compound A, deuterated compound A and compound B were carried out by liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) using an API 2000 mass spectrometer (PE Sciex, Ontario, Canada) by multiple reaction monitoring in the positive ion mode. The Turbo-IonSpray interface was used as the source of ionization. The high performance liquid chromatography (HPLC) system consisted of two Perkin Elmer series 200 micropumps (Boston, MA) and chromatography was carried out on a BetaBasic 5 μm C18, 4.6 × 100 mm column (Keystone, PA, USA). The mobile phase consisted of water containing 0.1% formic acid (solvent A) and acetonitrile containing 0.1% formic acid (solvent B). Linear gradient elution was carried out from 40% solvent B to 70% solvent B during a period of 7 min at a flow rate of 0.6 ml/min. The accuracy of quality control data was between 90% and 110% and precision was less than 10%.

Calculation of Pharmacokinetic Parameters

The pharmacokinetic parameters were calculated by established noncompartmental methods. The area under the

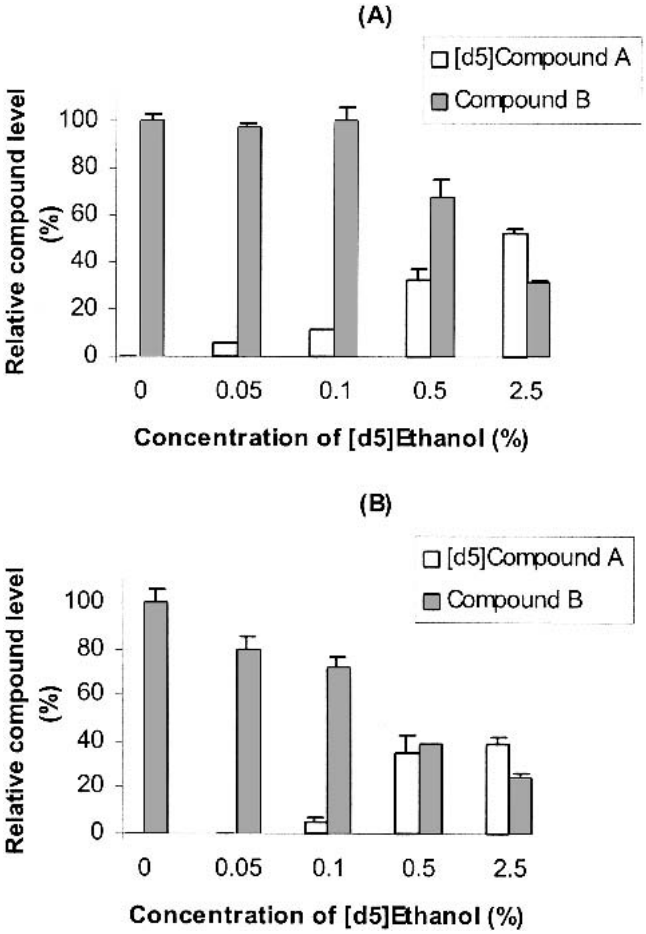

Fig. 2. Effect of [d_5]ethanol on the hydrolysis and trans-esterification of compound A in (A) rat plasma and (B) rat liver microsomes. Procedural details are described in "Materials and Methods." Data points, mean ± standard deviation for triplicate determinations, expressed as a percentage of the mean value of the group exhibiting the highest concentration. [d_5]Compound A is the product of trans-esterification of compound A, and compound B is the product of hydrolysis of compound A.

Table I. Trans-Esterification and Hydrolysis of Compound A in Rat Plasma and Rat Liver Microsomes Following Incubation at 37°C in the Absence or Presence of BNPP

Treatment	Plasma	Liver microsomes
Trans-esterification product ([d_5]compound A, μg/ml)		
–BNPP[a]	0.43 ± 0.13[b]	4.6 ± 0.24[b]
+BNPP[a]	0.09 ± 0.09[b]	0.01 ± 0.001[b]
Hydrolysis product (compound B, μg/ml)		
–BNPP[a]	22 ± 1.3[b]	2.5 ± 0.10[b]
+BNPP[a]	0.03 ± 0.01[b]	0.001 ± 0.001[b]

Trans-esterification was measured by the formation of [d_5]compound A. BNPP, bis(*p*-nitrophenyl) phosphate.
[a] Mean ± SD; n = 3.
[b] $p < 0.01$ between groups in the absence or presence of BNPP using Student's *t* test.

plasma concentration vs. time curve (AUC) was determined using Watson software (version 6.2.0.02; PSS, Inc., Wayne, PA, USA), with linear trapezoidal interpolation in the ascending slope and linear-log linear trapezoidal interpolation in the descending slope. The portion of the AUC from the last measurable concentration to infinity was estimated by the formula C_t/k_{el}, where C_t represents the last measurable concentration and k_{el} is the elimination rate constant. The latter was determined from the concentration vs. time curve by linear regression at the terminal phase of the semi-logarithmic plot. CL_p = Dose/AUC.

V_d = Dose × AUMC/AUC^2, where AUMC is first moment of the plasma concentration vs. time curve. Statistical significance of data between two groups was evaluated using Student's *t* test.

RESULTS

The schematic presentation of the hydrolysis and trans-esterification of compound A is shown in Fig. 1. The effects of deuterated ethanol on both the hydrolysis and the trans-esterification of compound A in rat plasma and hepatic microsomes are illustrated in Fig. 2. The amount of deuterated compound A (the trans-esterified product) generated increased with the increase in the amount of deuterated ethanol in incubates and was accompanied by the decrease of the amount of hydrolyzed product (compound B).

Mean concentrations of trans-esterified compound A ([d_5]compound A) were 0.43 ± 0.13 and 4.6 ± 0.24 μg/ml for rat plasma and rat liver microsomes, respectively, in the absence of BNPP (Table I). However, in the presence of BNPP, these values declined to 0.09 ± 0.09 and 0.01 ± 0.001 μg/ml for plasma and liver microsomes, respectively. The mean values for the hydrolysis product (compound B) were 22 ± 1.3 and 2.5 ± 0.10 μg/ml for plasma and liver microsomes, respectively, in the absence of BNPP (Table I); however, in the presence of BNPP, these values were 0.03 ± 0.01 and 0.001 ± 0.001 μg/ml, respectively.

Rats pretreated with ethanol exhibited elevated and prolonged concentrations of compound A in plasma as compared to water pretreated control rats (Fig. 3). The pharmacokinetic parameters for compound A following an intravenous dose, with or without oral pretreatment with ethanol, are given in Table II. The mean plasma clearance (CL_p) values were 860 ± 236 and 540 ± 185 $ml \cdot min^{-1} \cdot kg^{-1}$, the mean apparent volume of distribution (V_d) values were 13 ± 4 and 13 ± 7 L/kg, and the mean elimination rate constant (k_{el}) values were 0.048 ± 0.003 and 0.0253 ± 0.01 min, respectively, in the absence or presence of oral pretreatment with ethanol.

The amount of compound A that underwent trans-esterification was estimated using the equation: % trans-esterification = 100 × [$AUC_{[d5]compound\ A}/AUC_{compound\ A(total)}$], where $AUC_{compound\ A(total)}$ and $AUC_{[d5]compound\ A}$ represent the area under the plasma concentration curve for total compound A, which includes both compound A and deuterated compound A, and deuterated compound A, respectively, following an intravenous dose of compound A administered after oral pretreatment with deuterated ethanol. The mean AUC value for total compound A was 139 ± 26 ng · h/ml, whereas the mean AUC for [d_5]compound A was 41 ± 2.8 ng·hr/ml (Table II). Thus, approximately 29% of the intravenous dose of compound A underwent trans-esterification, assuming that the plasma clearance of deuterated compound A was identical to that of compound A.

DISCUSSION

In the GHS program, it was observed that a series of small molecule esters were readily hydrolyzed to form the corresponding acids in rodent plasma and rat liver microsomes (unpublished data). In the current set of experiments, deuterated ethanol was used to study the phenomenon of trans-esterification and to distinguish the ethyl ester of the

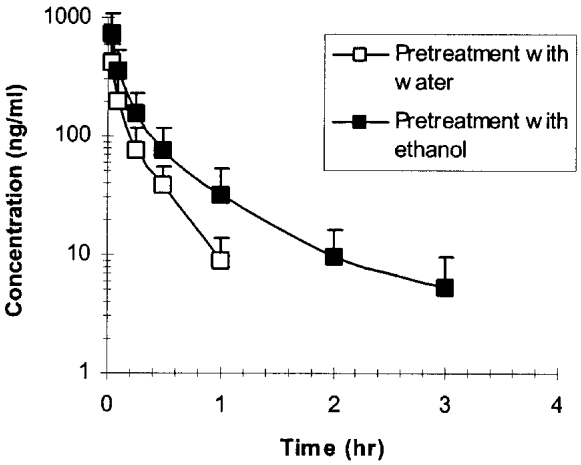

Fig. 3. Mean concentrations of compound A in the plasma of rats following an intravenous dose of compound A (5 mg/kg) after pretreatment with water or ethanol (3 g/kg). Data points are mean ± standard deviation for six rats.

Table II. Pharmacokinetic Parameters for Compound A (5 mg/kg) to Rats Intravenously Administered, Following Pretreatment With an Oral Dose of Water or Ethanol (3 g/kg)

Parameter	Administration of water before compound A (control)[a]	Administration of ethanol before compound A[a]
CL_p (ml·min^{-1}·kg^{-1})	860 ± 236[b]	540 ± 185[b]
V_d (L/kg)	13 ± 4	13 ± 7
k_{el} (min^{-1})	0.048 ± 0.003[b]	0.0253 ± 0.01[b]
Compound A (including deuterated compound A)		
$AUC_{\text{compound A (total)}}$ (ng·h/ml)		139 ± 26
Deuterated compound A formed *in vivo*		
$AUC_{\text{[d5]compound A}}$ (ng·h/ml)		41 ± 2.8
% Trans-esterification		29

[a] Mean ± SD, n = 6.

[b] $p < 0.05$ between the control and ethanol pretreated groups using Student's *t* test.

starting compound from that formed by the trans-esterification reaction. *In vitro* results obtained with rat plasma and rat liver microsomes showed that the amount of deuterated compound A formed (i.e., trans-esterified product) increased as the amount of deuterated ethanol was increased in the incubations. Furthermore, the amount of acid formed by hydrolysis (i.e., compound B) decreased with increasing concentrations of deuterated ethanol in the incubates. The results indicated that transesterification of compound A occurred in both rat liver and rat plasma. In separate experiments, compound A was not detected following incubation of compound B with ethanol and rat liver microsomes or rat plasma (data not shown). BNPP, a known carboxylesterase inhibitor, inhibited both the hydrolysis and the trans-esterification of compound A, simultaneously, implying that the same enzyme may be responsible for both reactions (8). These results supported the theory that water and ethanol both compete for acyl-enzyme intermediate (8), resulting, in this instance, in the formation of the acid (i.e., hydrolysis to compound B) or the deuterated compound A (i.e., trans-esterification).

Trans-esterification has been reported to occur *in vivo,* most notably with cocaine and with meperidine and methylphenidate (3–6). This phenomenon was investigated in rats following an intravenous administration of compound A, proceeded by an oral gavage of ethanol. The detection of deuterated compound A in the plasma samples demonstrated clearly that the trans-esterification of compound A indeed occurred *in vivo* in these rats. Furthermore, these results showed that the mean values of plasma clearance of compound A was significantly decreased by ethanol. The presence of ethanol provided an alternate pathway of metabolism (trans-esterification) and prolonged the ultimate hydrolysis of compound A, thereby decreasing the plasma clearance.

The extensive and rapid metabolism of ethanol *in vivo* ensured a transitory effect on the pharmacokinetics of compound A following a single oral dose of ethanol. It is likely, however, that the higher the levels of ethanol and the longer they are maintained, the more profound would be the effects on the pharmacokinetics, potentially altering the pharmacological effects of the compound. In conclusion, the results of our studies with compound A imply that the pharmacokinetic profiles of ethyl-ester drugs or prodrugs may be altered when administered in the presence of ethanol due to trans-esterification. The slower clearance may result in an increase of drug concentrations in plasma thereby enhancing drug action and possibly adverse effects associated with the drug. However, for ester prodrugs, co-administration of ethanol may delay and subsequently attenuate the actions of the drug. Therefore, it would be relevant to investigate the effect of trans-esterification of ester drugs in humans, especially in the presence of ethanol.

ACKNOWLEDGMENTS

We thank Ms. CarolAnn Keohane for her excellent assistance in conducting the animal studies.

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