Further Investigation of Amantadine Disposition:

Acetylation and Secretion

by

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ABSTRACT

Amantadine is a cationic aliphatic primary amine eliminated by the kidneys, excreted predominantly unchanged into the urine, and undergoes limited metabolism. Renal tubule secretion has an important role in its elimination. We studied two aspects of amantadine disposition, firstly acetylation, by developing a model to induce the enzyme spermidine/spermine N^1-acetyltransferase (SSAT1) with N^1, N^{11}-diethylnorspermine (DENSPM) and alcohol (Alc) as representative agents reported to induce its activity, and secondly renal secretion, by studying the effect of intravenous bicarbonate infusion on its renal elimination. We drew two conclusions, firstly, longer exposure to Alc combined with DENSPM administration provided the greatest potentiation of SSAT1 enzyme activity than each agent alone, which indicates a high likelihood of synergy between Alc and DENSPM; and secondly, bicarbonate load administered to healthy male volunteers impairs amantadine renal secretion in the absence of a clinically important change in blood pH, serum creatinine concentration or urinary creatinine clearance.

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ABBREVIATIONS

ACA	N-acetylamantadine
Alc	ethanol
ALP	alkaline phosphatase
AST	aspartate transaminase
ABC	ATP binding cassette
BRI	Biopharmaceutical Research Inc.
C_0	extrapolated concentration to t = 0
Cl_p	plasma clearance
Cl_r	renal clearance
Cr	creatinine
CV	coefficient of variation
DD	day
DENSPM	N^1,N^{11}-diethylnorspermine
GLC	gas-liquid chromatography
GFR	glomerular filtration rate
h	hour
HPLC	high performance liquid chromatography
IBW	ideal body weight
i.p.	intraperitonial
i.v.	intravenous
IS	internal standard
k_{el}	elimination rate constant

kg	kilogram
LC/MS/MS	high performance liquid chromatography/mass spectrometry
LLOQ	lower limit of quantitation
MDR	multidrug resistant
MEOS	microsomal ethanol oxidizing system
µg	microgram
µm	micrometer
min	minute
mL	millilitres
MM	month
MRM	multiple reaction monitoring
MRPs	multidrug resistance-associated protein transporters
NAT	N-Acetyltransferase
NAT1	N-Acetyltransferase-1
NAT2	N-Acetyltransferase-2
NMN	N^1-methylnicotinamide
NPT1	1 sodium/phosphate co-transporter
OATs	organic anion transporters
OATPs	organic anion-transporting polypeptide family
OCTs	organic cation transporters
OCTN	organic cation transporter, novel type
PAH	*p* -aminohippurate
PEPT1	peptide transporters

PDZ	post synaptic density protein, drosophila disc large tumor suppressor, and zonula occludens-1 protein domains
QC	quality control
SLC22A	solute carrier 22A
SSAT1	spermidine/spermine N^1-acetyltransferase
TEA	tetraethylammonium
TMD	transmembrane domain
$t_{1/2}$	half life
V_d	apparent volume of distribution
Vol.	Volunteer

GENERAL INTRODUCTION

Amantadine hydrochloride is an achiral polycyclic aliphatic primary amine used as an antiviral drug for the prophylaxis against and symptomatic management of respiratory tract infections due to type A influenza viruses, and the treatment of Parkinsonism and drug-induced extrapyramidal disorders (Merrick and Schmitt 1973; Parkes 1974). Its adverse effects have been usually referable to the central nervous system and included insomnia, jitteriness difficulty concentrating and mental depression, and less commonly gastrointestinal side effects such as dyspepsia (Smorodintsev, Zlydnikov et al. 1970; Dolin, Reichman et al. 1982). It is indicated for all ages and it has been administered to patients orally, intravenously and by inhalation. Oral formulations as capsules, tablets, and syrup remain the most used clinically.

Amantadine is excreted predominantly unchanged into the urine and undergoes limited metabolism in man (Koppel and Tenczer 1985). Amantadine clearance by the kidney is well documented. However, the entire administered dose is not recovered in the urine unchanged. 5-15% of the administered dose was recovered in the urine as acetylamantadine (ACA) (Koppel and Tenczer 1985). Renal tubular secretion is important for the elimination of this drug (Aoki, Sitar et al. 1979), and varies with the age of the human subject (Gaudry, Sitar et al. 1993).

The studies described in this thesis evaluate the effect of bicarbonate on tubular secretion mechanism(s) and provide initial results from development of a rat model to study amantadine acetylation through induction of the intracellular enzyme SSAT1.

AMANTADINE PHARMACOKINETICS

1- Absorption

Amantadine is relatively completely absorbed, ranging from 55-90% in a manner that is independent of dose, renal function, and formulation, but at a rate that is somewhat less in healthy elderly men than in younger individuals (Aoki and Sitar 1988). Oral dosing is the most commonly used route of administration, but the gastrointestinal site of absorption has not been defined. However, as a basic drug, its absorption from the stomach is expected to be negligible.

Relative bioavailability was first estimated by (Bleidner, Harmon et al. 1965), who demonstrated recovery of 86 ± 9% (mean ± SD) of amantadine in urine 0-96 h after 2-4 mg/kg oral doses in 5 healthy human subjects. The bioavailability of amantadine administered orally is nearly complete, because the V_d, $t_{1/2}$, and Cl_r are within the same range for healthy volunteers of the same age receiving the similar oral and i.v doses (Aoki and Sitar 1988). Data on time to peak plasma concentration suggest that amantadine is more rapidly absorbed in healthy young adults than in healthy older men (Aoki and Sitar 1985). Peak plasma amantadine concentration was 1.8 (Aoki and Sitar 1985) and 1.5 (Hayden, Minocha et al. 1985) times greater in healthy older subjects than in young individuals. Peak plasma amantadine concentration was directly related to dose ingested in young healthy volunteers (Bleidner, Harmon et al. 1965). However a study by Aoki et al. demonstrated over a wider range than Bleidner et al. (1965) that relative bioavailability was independent of dose (Aoki, Sitar et al. 1979). In chronic renal insufficiency absorption $t_{1/2}$ ranged from 0.2-1.5 h (Horadam, Sharp et al. 1981) and in another study by Wu et al., 0.6-1.3 mg of amantadine was recovered in the stool

collected by 6 healthy mean and 13 with chronic renal failure from 0-72 h (Wu, Ing et al. 1982), suggesting that amantadine absorption is independent of renal function.

2- Distribution

Aoki et al. (1979) reported an inverse correlation between amantadine dose and apparent volume of distribution (V_d) in 13 young healthy subjects (r = -0.52) (Aoki, Sitar et al. 1979). The normal V_d is 4.4 ± 0.2 L/kg, while in renal failure, it is 5.1 ± 0.2 L/kg (Soung, Ing et al. 1980). The fact that the V_d for amantadine exceeds body volume by a considerable amount suggests extensive tissue binding, which is expected for basic drugs (Aoki and Sitar 1988). Females have higher V_d values than male subjects, and elderly males have a smaller V_d than their younger counterparts (Aoki and Sitar 1985). The V_d after infusion of amantadine in healthy young volunteers yielded data similar to those derived after an oral dose.

In one study using blood bank samples, investigators found amantadine in plasma was approximately 67% protein bound, and the percentage bound was independent of drug concentration between 100 and 2000 μg/L. Mean total plasma protein concentration in these samples was 61 ± 5g/L, with 35 ± 4 g/L as albumin (Liu, Cheng et al. 1984). Another study reported amantadine plasma protein binding of 59% in 4 male hemodialysis patients using an ultrafiltration method. In these samples, total plasma protein concentration was 42 ± 3 g/L with plasma globulin concentration of 26 ± 4 g/L (mean ± SD). The major plasma binding protein for amantadine remains to be determined (Ing, Cheng et al. 1984). Daugirdas et al. reported substantial sequestration of amantadine by red blood cells, with higher erythrocyte: plasma ratios in normal healthy males than in impaired renal function and hamodialysis male patients

(Daugirdas, Ing et al. 1984). Very little data are available in humans concerning the extravascular distribution of amantadine. It was reported that amantadine appeared in saliva (Bleidner, Harmon et al. 1965), nasal mucus (Hayden, Minocha et al. 1985) and cerebrospinal fluid (Fahn, Craddock et al. 1971). Aoki & Sitar (1988) calculated amantadine concentration/g organ weight using average mouse organ weights method (Crispens and Marion 1975). This reanalysis demonstrated a lung to blood concentration ratio of 29 and a heart to blood concentration ratio of 6, one hour after a 1.6 mg/kg dose. The kidney also concentrated amantadine with a tissue to blood ratio of 29, one hour after the drug dose. The liver to blood ratio was lower at about 19, but its mass makes it the most important tissue quantitatively for the sequestration of amantadine. The calculated spleen to blood concentration ratio was about 11 (Aoki and Sitar 1988).

3- Renal Elimination

Renal clearance (Cl_r) is a dynamic process expressed as the sum of the rates of glomerular filtration (GFR) and tubular secretion minus the rate of tubular reabsorption. The filtration process is passive and only small unbound drugs can be filtered (less than 400-600 Å in diameter, about 5 kDa molecular weight) (Perri, Ito et al. 2003). Creatinine clearance (Cl_{cr}) is an accepted estimate of GFR. However it doesn't account for tubular secretion or reabsorption of drugs. The Cockcroft and Gault method has been the most often used bed side predictor of true 24 h measures of Cl_{cr} (Cockcroft and Gault 1976).

Amantadine, an organic cation drug, is eliminated from the body primarily by the kidney, and renal tubule secretion is important in this process. An average of 86% ± 9% of an orally administered dose is recovered in the urine unchanged in a collection period

that lasted 96 h in 13 healthy young adults after long-term dosage. A 1-compartment open model and complete bioavailability were assumed (Bleidner, Harmon et al. 1965). Elimination of the drug from plasma was consistent with a first-order process and plasma half-life ($t_{1/2}$) ranged from 10.2 to 31.4 h. The ratio of renal clearance:creatinine clearance (Cl_r:Cl_{cr}) ranged from 1.26 to 14.97, suggesting substantial renal tubular secretion (Aoki, Sitar et al. 1979). This interpretation is also supported by the observation that the ratio of the mean Cl_r of amantadine to mean total body clearance was 0.79 (Wu, Ing et al. 1982). Renal amantadine clearance exceeded glomerular filtration rate by 5 fold, and a median amantadine:creatinine renal clearance ratio of 4.20 in healthy young adults inferred that renal tubular secretion was important for the elimination of this drug (Aoki and Sitar 1988).

Several reports indicate that impaired renal function is a risk factor for amantadine accumulation and toxicity (Alvan, Kugelberg et al. 1980). The elimination $t_{1/2}$ in 6 subjects with normal renal function was 11.8 ± 2.1 h range (9.7-14.5 h) (Horadam, Sharp et al. 1981). In eight patients with various degrees of renal insufficiency, Cl_r ranged from 43.1-5.9 mL/min/1.73 m^2 and elimination $t_{1/2}$ values ranged from 18.5 h to 33.8 days. Also, 10 patients on chronic hemodialysis were studied, and the mean elimination $t_{1/2}$ during chronic hemodialysis was 8.3 days, and ranged from 7.0-10.3 days (Aoki and Sitar 1988).

The aging process is associated with prolongation in amantadine plasma $t_{1/2}$ and reduction in renal elimination (Aoki and Sitar 1988). In healthy elderly men, 60-76 years old, the plasma $t_{1/2}$ ranged from 18.5– 45 h with a mean of 28.9 h (Aoki, Sitar et al. 1979). Also, amantadine Cl_r is greater in males than females (Gaudry, Sitar et al. 1993).

Renal tubular secretion

Although GFR is about 120 mL/min in adults, reabsorption leads to only 1-2 mL/min of the filtered water load eliminated as urine. Renal transporters play an important role in importing, exporting, and exchanging a wide range of endogenous and exogenous substrates, affecting absorption, tissue distribution, and renal elimination of many drugs (Choi and Song 2008). In renal transport, the molecule first must pass from extracellular fluid into the renal tubular cell, and then into the tubular lumen. Thus there are distinct transporters, some at the basolateral membrane of the tubular cell and others at the apical bush border (Perri, Ito et al. 2003).

Amantadine is a cationic drug that is secreted by organic cation transporters (OCTs). To be able to understand organic cation renal tubular secretion, we need to give a general background about the transporters.

Transport across the cell membrane is mediated by specialized membrane proteins called transporters. Transporters are essential for vital processes like entry of all essential nutrients into the cytoplasmatic compartment and distribution of cellular products into and beyond the cellular membrane. These transporters are located in the epithelial membrane of the liver, kidney, intestine, and other target organs (Ciarimboli 2008).

Drugs are organic cations, organic anions, or neutral. Accordingly there are different types of transporters.

1- Organic anionic transporter family (OATs). The first, *p*-aminohippurate (PAH)/dicarboxylate exchanger was isolated from rat kidney and was called OAT1

(rOAT1) by the researchers (Sekine, Watanabe et al. 1997). Four human variants of OAT exist: hOAT1, hOAT2, hOAT3, and hOAT4 (Bahn, Prawitt et al. 2000).

2- Organic cation transporters family (OCTs). These include the sodium independent OCT 1, 2, and 3, and organic cation transporter, novel, type 1 and 2 (OCTN1 and OCTN2), which transport neutral and cationic hydrophobic compounds (Tamai, Yabuuchi et al. 1997).

3- Organic anion-transporting polypeptide family (OATPs). In all species, this subfamily of transporters comprises at least 14 members (Kullak-Ublick, Hagenbuch et al. 1995).

4- The multidrug resistance-associated protein transporters (MRPs) family is the third subgroup of the ATP binding cassette (ABC) transporter super family. It comprises 13 members, ABCC1 to ABCC13 (Choudhuri, Ogura et al. 2001).

Renal tubular secretion of drugs is a very active field of research and ongoing investigations are discovering new transporters, such as type 1 sodium/phosphate co-transporter (NPT1). Particular regions where protein-protein interactions occur, which consist of three proteins (post synaptic density protein, drosophila disc large tumor suppressor, and zonula occludens-1 protein) are known as PDZ domains (Fanning and Anderson 1999; Gisler, Stagljar et al. 2001), peptide transporters (PEPT1 and PEPT2) with 50% homology (Leibach and Ganapathy 1996), and prostaglandin transporters (Pucci, Bao et al. 1999). In our human study, we were interested in OCTs, as amantadine is an organic cation that is believed to be secreted through them.

Organic cationic transporters (OCTs)

As more than 30% of clinically used drugs are organic cations, drug interactions between substrates and/or inhibitors of OCTs are quite likely (Hung, Chang et al. 2002). Rat organic cation transporter 1 (OCT1) was first cloned in 1994 (Grundemann, Gorboulev et al. 1994). Later other members of the OCT family have been discovered, including OCT2, and OCT3. They are organized in the SLC22A family, and show a similar membrane topology consisting of 12 transmembrane domains (TMDs), an intracellular N-terminus, a large glycosylated extracellular loop between TMDs 1 and 2, a large intracellular loop with phosphorylation sites between TMDs 6 and 7, and an intracellular C-terminus (Koepsell, Lips et al. 2007). A variety of endogenous and exogenous compounds, in addition to some therapeutic drugs are secreted through OCTs.

Examples of OCTs substrates, include model organic cations such as tetraethylammonium (TEA) and decynium 22, clinically important therapeutic drugs, e.g. metformin, procainamide, cisplatin, citalopram, and cimetidine, endogenous compounds, such as dopamine and norepinephrine, and toxic substances such as neurotoxic pyridinium metabolites (Choi and Song 2008).

OCT1 is primarily expressed in the sinusoidal membrane of hepatocytes and is also present in the epithelial membrane of the intestine at a low level, while OCT2, the most abundant organic cation transporter (Urakami, Okuda et al. 1998), is primarily expressed in the basolateral membrane of the kidney proximal tubules. OCT3 shows a widespread tissue distribution, including the brain, heart, skeletal muscle, blood vessels, placenta, and liver (Koepsell, Lips et al. 2007). Genetic factors are believed to account

for much interindividual variability in drug disposition and effect. Knockout mouse models have been generated for OCT1, OCT2, and OCT3, and genes Slc22a1, Slc22a2, and Slc22a3 respectively (Choi and Song 2008). Combined deficiency of OCT1 and OCT2 better reflects the effect of OCT2 deficiency on kidney function in humans (Jonker and Schinkel 2004). The accumulation of one neurotoxic pyridinium metabolite in the heart and foetus is significantly reduced in OCT3 deficient mice compared to wild-type mice (Choi and Song 2008). These knockout animal studies emphasize the role of OCTs in the hepatic and renal elimination and tissue distribution of substrates.

Regulation mechanisms of OCTs are important because they change the mRNA or protein level of OCTs, and as a result affect their substrates distribution. There are exogenous and endogenous factors, such as activators or inhibitors of protein kinase A (PKA), Src-like p56, calmodulin (CaM), HNF-4a, 1,2-diocanoyl-sn-glycerol (DOG), and pregnenolone-16a-carbonitrile (PCN) that have been demonstrated to modulate OCT activity (Choi and Song 2008). Recently several studies reported polymorphic variation and several single nucleotide polymorphisms (SNPs) for SLC22A. It was found that the allele frequencies of non-synonymous SNPs in SLC22A1 and SLC22A2 genes are different among different ethnic groups, which may help to understand and explain variation of drug pharmacokinetics among them (Kerb, Brinkmann et al. 2002).

Amantadine renal tubular secretion

Our lab has been involved in several experiments studying the role of amantadine secretion through OCTs. Amantadine is a clinically important achiral organic cation with a pKa of 10.1, and its ionization is not dramatically changed around

physiologic pH (Aoki and Sitar 1988). With increased age, amantadine Cl_r is reduced disproportionately to Cl_{cr} (Aoki and Sitar 1985) and amantadine Cl_r is greater in men than in women (Wong, Sitar et al. 1995). Case reports indicate clinically significant drug interactions for renal elimination between amantadine and other organic cations such as quinine and quinidine (Gaudry, Sitar et al. 1993). Amantadine renal tubular excretion is affected by cimetidine , nicotine, cotinine and ouabain (Wong, Smyth et al. 1992; Escobar and Sitar 1996). A study demonstrated reduced expression of rOCT1 and rOCT2 in the chronic diabetic state, and a restoration in their levels by exogenous insulin (Grover, Buckley et al. 2004). Another study demonstrated that amantadine transport increased while TEA transport decreased in kidney tissue from acutely diabetic rats compared with that from control rats (Goralski, Stupack et al. 2001; Grover and Atwal 2002). For both substrates, transport perturbations were reversed by exogenous insulin, implicating the diabetic state as the responsible mechanism.

Another important focus of our research is the contrasting roles for bicarbonate on renal tubular amantadine transport. When human volunteers taking oral amantadine were given chronic oral bicarbonate, a decrease in amantadine excretion followed by an increase in plasma amantadine concentration was observed (Geuens and Stephens 1967). From their study, it was inferred that bicarbonate decreased amantadine excretion by increasing urine pH and thus increasing its passive reabsorption. In contrast, *in vitro* rat experiments have demonstrated that at constant pH, the energy-dependent uptake of the organic cation amantadine into proximal and distal tubules is primarily mediated by bicarbonate-dependent transport sites (Escobar, Wong et al. 1994; Escobar and Sitar 1995), which might indicate that bicarbonate exerts its effects at both the basolateral and

brush border membrane, and that its effects are opposite at the two sites. *In vivo* experiments in rat assessed the functional significance of *in vitro* study findings. Elevation of plasma bicarbonate by 10 mM after acute i.v. bicarbonate dose was associated with reduced amantadine Cl_r which returned to normal shortly after plasma bicarbonate concentration returned to control values (Aoki and Sitar 1988; Goralski, Smyth et al. 1999). Continuous bicarbonate infusion to anesthetized dog eliminated renal amantadine secretion, while creatinine excretion was essentially unaffected (Sitar, Escobar et al. 1997), indicating that the inhibitory effect is not species specific.

In our current human study, we were interested in investigating the applicability of the previous *in vitro* and *in vivo* rat and dog studies to humans. We hypothesized that bicarbonate administered to healthy male human volunteers will impair amantadine renal secretion in the absence of a clinically important change in blood pH, serum creatinine concentration and urinary creatinine clearance.

4- Metabolism

Early studies reported that humans didn't metabolize amantadine. These workers stated that there was no evidence for acetylated or methylated metabolites of this drug in humans, but their metabolic methods were not very sensitive (Bleidner, Harmon et al. 1965). Some studies demonstrated incomplete urinary recovery of an orally administered dose and the cause was assumed to be due to incomplete absorption (Aoki, Sitar et al. 1979; Horadam, Sharp et al. 1981). Wu et al. 1982 found 0.6 to 1.3mg amantadine in stools collected from 0-72 h after ingestion of amantadine 100 mg, suggesting the that excretion of undetected metabolites might be the reason for incomplete urinary recovery

(Wu, Ing et al. 1982). In a case of amantadine overdose, several metabolites were identified by gas chromatography/mass spectrometry. This finding led to the re-investigation of the metabolism of amantadine under a therapeutic dosing regimen (Fahn, Craddock et al. 1971). It was found that the bulk of the dose was eliminated unchanged but 5-15% of an administered dose undergoes N-acetylation, which is the major metabolic pathway that converts amantadine from a base to a weak acid. However, several other unusual metabolic pathways were observed such as: N-methylation, formation of Schiff bases and N-formiates. No metabolites with a hydroxylated adamantane ring system could be detected (Koppel and Tenczer 1985). Several studies which will be discussed below have been conducted to evaluate the expected acetylation of amantadine, which was not unusual because of the presence of a primary amino group.

Metabolism by acetylation

Acetylation describes a reaction that introduces an acetyl functional group for an active hydrogen atom in an organic compound. It can occur for endogenous or exogenous compounds in the human body. Acetylation as a phase II drug metabolism pathway has been recognized for many years (Weber and Hein 1985). Williams stated that acetylation could only be considered a general reaction of amino groups attached to an aromatic ring. However, he acknowledged that acetylation of aliphatic amino group had also been observed (Williams 1959b). It was soon realized that drug acetylation is under genetic control (Evans, Manley et al. 1960) and occurred by N-acetyltransferase enzymes.

N-acetyl transferases NAT1 and NAT2

Arylamine *N*-acetyltransferases (NATs); N-acetyltransferase 1 (NAT1) and N-acetyltransferase 2 (NAT2) have had historic roles in cellular metabolism, carcinogenesis, and pharmacogenetics. They catalyze the detoxification of arylamines by N-acetylation and the bioactivation of *N*-arylhydroxylamines by O-acetylation (Sim, Westwood et al. 2007).

Jenne was the first to suggest that two acetylation pathways exist in man, using human liver tissue from fast and slow acetylators. She also suggested that the differences in acetylation activity were due to a difference in the amount of enzyme present in fast and slow acetylators (Jennne 1965). Isoniazid (INH) induced nerve damage in "slow" activators (acetylators) of INH and led to the discovery of the human acetylation polymorphism (Weber and Hein 1985). Grant et al. showed that the slow acetylation both *in vivo* and *in vitro* was the result of a decrease the quantity of NAT protein in slow acetylators relative to fast acetylators (Grant, Morike et al. 1990).

Human NAT1 and NAT2 are non inducible enzymes with 81% identical sequences, and both exhibit genetic polymorphism. Over 25 human NAT1 and NAT2 alleles have been identified. The ability to rapidly acetylate drugs is inherited as an autosomal dominant trait, and is found in different frequencies in different ethnic groups (Hein, Doll et al. 2000). Associations between the slow acetylator phenotype and adverse drug reactions have been found (Weber and Hein 1985). Many drugs are metabolized by acetylation in man, including procainamide, isoniazid, and sulfonamides, which make acetylation enzymes an important field of research.

Amantadine acetylation

Amantadine contains an aliphatic amine group and was expected to be acetylated by NAT1 and/or NAT2. However, a study was conducted to confirm that amantadine is acetylated in humans and examined for the first time whether the extent was correlated with NAT2 acetylator phenotype. Thirty-eight normal, healthy volunteers were NAT2 acetylator phenotyped with sulfapyridine. There was no correlation between NAT2 acetylator phenotype and amantadine acetylation ($P < 0.5$), and no difference in the total urine amantadine excreted over 8 h between rapid acetylators and slow acetylators (28.3 ± 9.7 mg) versus (30.4 ± 9.6 mg) respectively (mean ± SD). Similar *in vitro* enzyme studies demonstrated that neither NAT1 nor NAT2 was responsible for acetylation of amantadine. There is no evidence that amantadine is able to inhibit acetylation of the prototypical NAT1 and NAT2 substrates *p*-aminobenzoic acid and sulfamethazine by any of the mammalian enzyme sources tested. As well, these enzymes were unable to acetylate amantadine, even in the absence of *p*-aminobenzoic acid and sulfamethazine (Bras, Hoff et al. 1998).

Subsequently, another study investigated whether the inducible enzyme spermidine/spermine N^1-acetyltransferase (SSAT1) was the responsible enzyme for amantadine acetylation. Amantadine acetylation was demonstrated both *in vivo* and *in vitro* using transgenic male mice overexpressing SSAT1. Transgenic mice injected s.c. with 3 mg/kg amantadine excreted 4.5 ± 1% (mean ± S.E.) of the administered dose as acetylamantadine in 24 h urine samples while, in contrast, nontransgenic control mice failed to excrete any detectable acetylamantadine in their urine. *In vitro* studies with the cytosolic liver fraction from transgenic mice as the source of SSAT1 demonstrated that

amantadine competitively inhibited spermidine acetylation with an apparent K_i of 738 ± 157 μM. The NAT2 substrate, sulfamethazine, inhibited spermidine acetylation with a calculated *K*i of 3.5 mM, suggesting that SSAT1 may be an alternate pathway for acetylation of NAT2 substrates. The NAT1 substrate, *p*-aminobenzoic acid, had no inhibitory effect. These results provide evidence that amantadine can be acetylated by SSAT1 and may be a specific drug substrate for this enzyme (Bras, Janne et al. 2001).

The previous two studies and other reports that ethanol (Alc) ingestion induced some arylamines acetylation raised the possibility that SSAT1 enzyme might be responsible for acetylation of drugs other than amantadine. It was reported that Alc ingestion increased INH acetylation in both humans and rats (Lester 1964; Thomas and Solomonraj 1977). Also, it was reported that slow and fast drug acetylators excreted an increased fraction of ingested sulfadimidine and procainamide as acetylated metabolites when they ingested Alc concurrently. A suspension of isolated rat liver cells showed an increase by about 30% in the rate of sulphadimidine acetylation after the addition of Alc (Olsen and Morland 1978; Olsen and Morland 1982). Since it is believed that NAT2 is the enzyme responsible for those drug conjugation reactions, and NAT enzymes are not inducible, Olsen and Morland speculated that another pathway uninfluenced by acetylator phenotype or Alc could explain their experimental observations.

SSAT1 is present in very small amounts in cells and needs to be induced for further metabolism studies (Fogel-Petrovic, Kramer et al. 1997). The models to study SSAT1 enzyme are expensive, such as supernatant from homogenized liver, human enzymes transfected into E coli bacteria, and transgenic mice or rats overexpressing SSAT1 (Perin and Sessa 1993; Bras, Hoff et al. 1998). Also, the transgenic mouse

model overexpressing SSAT1 had many toxic effects characterized by substantial alteration of polyamine pools, hair loss, female infertility, weight loss, and altered lipid metabolism (Pegg, Feith et al. 2003).

In our present study, we attempted to develop a safe, cheaper and effective rat model to induce SSAT1 enzyme. To be able to choose agents to induce SSAT1 we need to understand its biology and kinetics.

Spermidine/spermine N^1-acetyltransferase (SSAT1)

The N^1-acetylation of polyamines spermidine and spermine by SSAT1 is a crucial step in the regulation of cellular polyamine levels in eukaryotic cells, and it is localized to the mitochondria, but it could also be found in the cytoplasm and the nucleus (Holst, Nevsten et al. 2008). The polyamines, spermine, spermidine, and their diamine precursor, putrescine, are naturally occurring polycations that play an important role in numerous physiological functions such as cell growth and proliferation, nucleic acid and protein synthesis, cell adhesion and repair of the extracellular matrix, and immunity (Pegg 1986; Hegde, Chandler et al. 2007). Altered polyamine levels are associated with a variety of cancers as well as other diseases.

The first regulatory step in the polyamine biosynthetic pathway is the conversion of ornithine to putrescine by ornithine decarboxylase. The successive polyamines, spermidine and spermine, are then synthesized by the sequential addition of aminopropyl groups to putrescine, catalyzed by their respective synthases. Then SSAT1 acetylates spermine and spermidine, and the monoacetylated spermidine and spermine are either degraded by N^1-acetylpolyamine oxidase or exported from the cell. A very

small amount of SSAT1 is present in the liver, even after maximal induction (about 9 ng/mg of soluble protein), which corresponds to only about 60,000 molecules per hepatocyte (assuming that the enzyme is confined to these cells) (Ragione and Pegg 1982).

SSAT1 is an unstable enzyme with a short half life ($t_{1/2}$ = 29 min) (McCloskey, Coleman et al. 1999) and is rapidly induced by a variety of stimuli such as heat shock, hormones and growth factors, toxic compounds, polyamines and polyamine analogues, certain drugs, and pathophysiological conditions (Matsui and Pegg 1981; Casero and Pegg 1993; Thomas and Thomas 2003). SSAT1 may be increased up to 50-100-fold by maximal induction (Pegg, Borchardt et al. 1981). Subsequently, another SSAT was discovered by homology search (SSAT2) (Chen, Vujcic et al. 2003), with 61% homology with SSAT1. SSAT2 is not inducible by polyamines and not involved in intracellular polyamine pools regulation (Coleman, Stanley et al. 2004). SSAT1 amino acid sequences for mouse, hamster, and human have a homology of greater than 96% (Pegg, Stanley et al. 1992; Fogel-Petrovic, Kramer et al. 1993). Thus any finding in the rat model could be expected to be important in man.

Human cancer patients excrete an increased amount of polyamines and their metabolites in urine (Russell, Levy et al. 1971; Suh, Lee et al. 1997), which leads to the rationale for studying the key enzymes in the polyamine pathway, including SSAT1, as potential therapeutic drug targets. Polyamine analogs have been actively investigated for their potential therapeutic relevance, because they down regulate enzymes involved in the synthesis of polyamines, while stimulating enzymes implicated in the catabolism of polyamines, such as SSAT1 and polyamine oxidase (Mitchell, Leyser et al. 2002). One

of polyamines most powerful SSAT1 inducer is N^1, N^{11}-diethylnorspermine (Fogel-Petrovic, Kramer et al. 1997).

N^1, N^{11}-diethylnorspermine (DENSPM)

DENSPM is a first generation alkylated analog of spermine that had demonstrated encouraging antitumor activity in several preclinical models. The *in vitro* effect of DENSPM on human melanoma cells includes a 980 fold increase in SSAT1 activity, an increase in SSAT1 $t_{1/2} > 12$ h, polyamine pool depletion, cell cycle arrest, and induction of apoptosis. (Porter, Ganis et al. 1991; Fogel-Petrovic, Kramer et al. 1997; Kramer, Fogel-Petrovic et al. 1997). This compound was also studied on different cancer cells, such as human breast cancer cells (Hegardt, Johannsson et al. 2002). In transgenic mice with overexpression of SSAT1, administration of DENSPM led to dramatic increases in SSAT1 activity and near total depletion of polyamine pools associated with greater toxicity (Alhonen, Pietila et al. 1999). In a Phase II study, DENSPM was used daily for 5 days every 21 days in patients with previously treated metastatic breast cancer (Wolff, Armstrong et al. 2003) and in a Phase I study in patients with non small cell lung cancer (Hahm, Ettinger et al. 2002).

Polyamines analogues appear to regulate SSAT1 $t_{1/2}$ by inhibiting ubiquitination of the enzyme, and thereby preventing its targeting proteosomal degradation (Coleman and Pegg 2001). These analogues efficiently reduce the polyamine pools, not only by stimulating degradation of the natural polyamines, but also by inhibiting their biosynthesis (Huang, Hager et al. 2003; Holst, Nevsten et al. 2008).

As DENSPM is regarded as the most potent known inducer of SSAT1, the safety preclinical toxicological studies that were carried out in rats and dogs determined its safe dose (Kanter, Bullard et al. 1994). It represented an appealing agent to be used in developing a rat model for SSAT1 induction in our project.

Ethanol (Alc)

Alc is also known to increase the intracellular level of SSAT1 in humans and rats (Casero and Pegg 1993; Perin and Sessa 1993). Alc is not a specific inducer of SSAT1. The acute interaction between Alc and drugs usually leads to a decreased rate of drug metabolism. Both oxidation and conjugation of drugs with glucuronic acid may be inhibited by Alc (Nelson 1962; Moldeus, Vadi et al. 1976). The activity of the microsomal ethanol-oxidizing system (MEOS) increases, with an associated rise in cytochromes P-450, especially the isoform CYP2E1 (Lieber 2004). As mentioned above, several reports indicated that Alc stimulated drug acetylation in human and rats (Lester 1964; Thomas and Solomonraj 1977; Olsen and Morland 1982). Alc also represents a commonly used agent by the public, cheap and easy to obtain, that can be used to induce SSAT1 in our rat model.

HYPOTHESES

As discussed above, amantadine is an organic cation, and factors that affect its secretion might also affect secretion of other organic cations. SSAT1 could be a potential acetylating enzyme for drugs other than amantadine. We were interested in this research program to study the effect of bicarbonate load on amantadine renal secretion and to build a more economical and practical animal model to induceSSAT1 using the

previously discussed agents DENSPM and Alc. In chapter one we tested the hypothesis that a bicarbonate load administered to 12 healthy human males will impair amantadine renal secretion in the absence of a change in serum creatinine concentration or urinary creatinine clearance.

In chapter two we proposed three hypotheses. First, chronic exposure to Alc in drinking water will increase SSAT1in rat liver, which will be reflected by the presence of N-acetyl amantadine (ACA) in rat urine. Second, chronic exposure to DENSPM will increase SSAT1 in rat liver that will be reflected by the presence of ACA in rat urine. Our last and third hypothesis proposed a synergistic effect between the two agents, Alc and DENSPM in inducing SSAT1.

1. CHAPTER 1: THE EFFECT OF BICARBONATE INFUSION ON AMANTADINE RENAL SECRETION

1.1. INTRODUCTION

Organic cation transporters (OCTs) are proteins that play an important physiological and pharmacological role in the reabsorption and/or secretion of positively charged endogenous and exogenous cationic compounds within the body. In the kidney, they are mainly expressed in the proximal renal tubule and in the collecting duct (Launay-Vacher, Izzedine et al. 2006). However, OCTs have also been characterized in the distal renal tubule, at least in rats (Escobar, Wong et al. 1994; Goralski, Lou et al. 2002).

Mechanisms for organic cationic transport across renal tubules have been extensively studied, but our understanding of these mechanisms remains considerably deficient. These organic cationic transport processes are saturable, energy dependent, and occur by a system separate from organic anion transport mechanisms (Ciarimboli and Schlatter 2005; Wright 2005). Amantadine represents a clinically relevant organic cation with interesting chemical and disposition characteristics compatible with its consideration as a prototypic probe to study renal clearance mechanisms for organic cationic drugs (Goralski, Lou et al. 2002). It is an achiral basic drug with a single functional amino group with a pKa of 10.1, that is virtually completely ionized at physiologic pH, and amantadine is both secreted and filtered by the kidney (Takano, Kitahara et al. 1984; Aoki and Sitar 1988).

An early study reported a decrease in amantadine urinary excretion after chronic oral bicarbonate, and this observation was explained by the effect of pH change on the

ionization of amantadine, ignoring any specific effect for bicarbonate ion (Geuens and Stephens 1967). Another study by Ullrich et al showed that the transport of NMN, a prototypical organic cation marker, is significantly inhibited by the absence of bicarbonate in an *in vivo* microperfused rat proximal tubule preparation (Ullrich, Rumrich et al. 1991). Subsequently, other studies suggested that the decrease in amantadine excretion might be due to a direct effect of bicarbonate ion on organic cation transporter function (Escobar, Wong et al. 1994; Sitar, Escobar et al. 1997; Goralski, Smyth et al. 1999; Gerlyand and Sitar 2009)

Disorders in which plasma bicarbonate concentration rises above normal are quite common in humans, and include metabolic alkalosis and metabolic compensation to respiratory acidosis (Williamson 1995). In addition, multiple chronic diseases, often involving the heart, lungs, and kidneys, can affect acid-base equilibrium (Dennis 1985) . Healthy older adults develop a chronic low-grade metabolic acidosis associated with a decrease in circulating bicarbonate and an increase in lactate concentration (Frassetto, Morris et al. 1996). Also, diabetes often is associated with acid-base disturbances and organic cation energy-dependant transport is upregulated within 4 days of induction of the disease in a rat model (Goralski, Stupack et al. 2001). These occurrences may represent pathological conditions in which organic cation elimination by the kidney may be compromised.

Renal tubular transporters play an essential role in drug urinary excretion. As a result, any alteration of their expression or activity may influence transport equilibrium from blood into renal tubular cells, and from renal tubular cells to the extracellular lumen. Possible outcomes include intracellular accumulation of drugs, altered

pharmacokinetic disposition, as well as alteration to their tolerance and efficacy profile. Furthermore drug interactions at one or several transporters may have similar consequences, such as renal toxicity or systemic drug accumulation, depending where the transporter interaction occurs (Launay-Vacher, Izzedine et al. 2006).

In the current study, we investigated the effect of changing bicarbonate concentration in the blood without changing the blood pH on amantadine renal excretion. As a difference in amantadine excretion between men and women has been reported (Wong, Sitar et al. 1995), we conducted the present study in healthy male volunteers to evaluate whether results would be consistent with our evolving understanding of mechanisms that may control the renal elimination of basic drugs. We hypothesized that a bicarbonate load administered to healthy male human volunteers will impair amantadine renal secretion in the absence of a clinically important change in blood pH, serum creatinine concentration or urinary creatinine clearance.

1.2. EXPERIMENTAL METHODS

1.2.1. Inclusion criteria for volunteers

Volunteer acceptance into the study required that they be male and that there was evidence of no clinical renal or cardiovascular disease. Good health was confirmed by history, physical exam (blood pressure, nose, throat, heart and lung examination) and from biochemical analysis of a blood sample for hemoglobin, white blood cell and platelet concentration, liver function tests (serum bilirubin, lactate dehydrogenase, aspartate transaminase (AST), alkaline phosphatase (ALP), and serum creatinine concentration as a measure of renal function.

Subjects were informed that side effects from amantadine ingestion are highly unlikely because only a single dose will be administered. Side effects may include a hypersensitivity response due to an immune mechanism, and any other side effects if they should occur would be most likely related to the effect of amantadine on the brain. These effects may include insomnia, jitteriness, and difficulty in concentrating and mental stimulation. However, these side effects are associated with chronic ingestion of amantadine and are highly unlikely to occur with ingestion of a single dose (Aoki and Sitar 1988).

1.2.2. Exclusion criteria for volunteers

Females were excluded from participating in this protocol. Any previous adverse reaction to amantadine excluded the person from volunteering for this study.

1.2.3. Experimental protocol

The protocol for this study is a balanced randomized, two-arm cross-over design, with at least one week separating each arm. The study was completed in 12 healthy male adult volunteers. Study approval was granted by the University of Manitoba Faculty Committee on the use of Human Subjects in Research. Informed signed consent was obtained from each participant (Approval B2006:179).

Volunteers were requested to abstain from alcohol ingestion for at least 48 h prior to each of the two study days, and not to ingest any other drugs as therapy. They ingested 3 mg/kg amantadine HCl syrup USP (10 mg/ml; Pharmascience Inc., Montreal, QC) at 2000 h the night before the study (2 h after supper), consistent with our previously completed protocols (Gaudry, Sitar et al. 1993; Wong, Sitar et al. 1995). The next morning, volunteers were allowed to have breakfast, and arrived at the Clinical Pharmacology Investigation Laboratory (Health Sciences Centre) at 0730 h to allow for the interventions described below to be completed. An intravenous (i.v.) cannula was placed in a forearm vein, and subjects emptied their bladder just before 0800 h. Diuresis was established by ingestion of a one liter bottled water load at 0800 h, and volume eliminated, as determined by total urine excretion during each 2 h collection period, was replaced by oral ingestion of an equal volume of bottled water or by the i.v. infusion between 1000 h and 1200 h. Blood samples (10 ml) were collected from the i.v. cannula every 2 h starting at 0800 h for determination of amantadine concentration in the plasma. An i.v. infusion of 2 mmol/kg lean body mass 0.9% w/v sodium chloride injection USP (Baxter Corp., Mississauga, ON) or an equivalent dose of hypertonic sodium bicarbonate (8.4% Sodium Bicarbonate Injection USP, Hospira Healthcare Corp, St.

Laurent, QC) in 5% Dextrose Injection USP (Baxter Corp., Mississauga, ON) (1 mmol/ml bicarbonate concentration in dextrose solution) was administered over a 2 h time interval from 1000 to 1200 h by infusion pump (Baxter model Colleague CX, Deerfield, IL, USA).

The two interventions were allocated on alternate study days. Serum and urine creatinine concentrations were measured concurrently as an indication of the maintenance of renal function. Renal clearance of amantadine and creatinine were calculated. This protocol mimics our previous clinical studies on renal clearance of amantadine and creatinine (Gaudry, Sitar et al. 1993; Wong, Sitar et al. 1995).

1.2.4. Sample Collection

1.2.4.1. Urine samples

Volunteers emptied their bladder prior to the commencement of sample collection at 0800 h. Complete urine samples were collected in 2 h intervals from 0800 to 1600 h (4 timed intervals). Volume and pH were measured, and aliquots within the times bracketed by the blood samples for determination of amantadine and creatinine were frozen and stored at -20°C until analyzed for amantadine by high performance liquid chromatography/mass spectrometry (LC/MS/MS). Biological specimens for analysis were coded so that the analyst was blinded to the treatment regimen. We have demonstrated previously stability of amantadine renal clearance determinations from quantitative 2 h urine specimens from human volunteers (Gaudry, Sitar et al. 1993; Wong, Sitar et al. 1995).

1.2.4.2. Plasma and serum samples

The i.v. catheter inserted into forearm vein of each volunteer prior to infusion was kept patent with 2 ml of bacteriostatic saline solution. Blood samples were collected from the i.v. cannula at 0800, 1000, 1200, 1400, and 1600 h. Before each sample was collected, 2 mL of fluid were removed and discarded from the i.v. catheter to ensure that the collected sample was not diluted with saline. The blood sample was placed into vacuum tubes containing 20 mg of potassium oxalate and 25 mg of sodium fluoride (grey-stoppered Vacutainer tubes, Becton Dickinson, Mississauga, ON) and mixed immediately. Plasma from each sample was immediately separated by centrifugation (1000 *x* g for 10 min) and frozen at -20°C for analysis by HPLC/MS/MS using selected ion monitoring (Biopharmaceutical Research Inc.(BRI), Vancouver, BC).

An additional 5 mL of blood was removed for the determination of serum creatinine, urea, glucose, sodium, potassium and chloride concentrations for each of the 5 time periods (Clinical Biochemistry Laboratory, Health Sciences Centre, Winnipeg, MB). An additional 200 μL of blood (1.0 ml in total each day) was removed into heparinized syringes (Westmed, Tucson, AZ, USA) to determine blood bicarbonate concentration (Critical Care Laboratory, Health Sciences Centre, Winnipeg, MB).

1.2.5. Analytical methods

1.2.5.1. Serum and urine creatinine analyses

The serum and urine creatinine were analyzed at the Health Sciences Centre Clinical Chemistry Laboratory using an enzymatic *in vitro* assay (CREA plus, Roche Diagnostics, Indianapolis, IN) for the direct quantitative determination of creatinine in human serum, plasma and urine using Roche clinical chemistry analyzers. For the urine samples no preservative is added and the urine is diluted with 10 volumes distilled deionized water before analysis.

The enzymatic method involves metabolism of creatinine to hydrogen peroxide. This process involves catalysis by creatininase, creatinase, and sarcosine oxidase. Hydrogen peroxide is measured via a modified Trinder reaction by its reaction with 4-aminophenazone and 2,4,6-triiodo-3-hydroxybenzoic acid. The color intensity is directly proportional to the concentration of creatinine present and is measured photometrically.

1.2.5.2. Amantadine analyses in human biological fluids

1.2.5.2.1. Plasma analysis

BRI developed a LC/MS/MS assay method for quantitation of amantadine in human plasma samples. This assay was a modification of a previously qualified assay for the determination of acetylamantadine and amantadine in human urine.

Frozen plasma samples (fluoride/oxalate anticoagulant) were received by BRI in 10 mL capped polypropylene vials on dry ice and then prepared for analysis after being thawed. In a 1.7 ml microcentrifuge tube, rimantadine HCl (10μL, 10 μg/ml), as an internal standard (IS) (Sigma-Aldrich Canada Ltd., Oakville, ON)) was added; then amantadine HCl working stock solution (10 μL), and human plasma (100 μL) were

added to the above tube followed by vortex mixing for approximately 30 sec. For protein precipitation trichloroacetic acid (150 μL, 5% w/v) (Sigma Aldrich Canada Ltd, Oakville, ON) in acetonitrile/water (20% v/v) was added to the tube followed by vortex mixing for 30 sec and centrifugation at 13000 x g for 5 min. The supernatant was transferred to 250 μL HPLC vials, capped and centrifuged at 3440 x g for 5 min, then analyzed by LC/MS/MS with an Agilent Model 1100G binary pump, Agilent Model 1100G column compartment, and an Agilent Model 1100 autosampler. Sample separation was accomplished with a Synergy Hydro-RP 80A 4 μm particle size (50 x 2.0 mm, id) column (Phenomenex, Torrence, CA), with tandem MS/MS detection using an electrospray ionization triple-quadrupole mass analyzer (Micromass Quattro®-LC triple quadrupole mass spectrometer). Column flow rate was (0.3 ml/min) and the retention times for the amantadine and rimantadine were 4.6 and 5.0 min respectively. Quantitation of amantadine in human plasma was performed based on the peak area response ratio of amantadine to the IS spiked at a constant level to all samples. HPLC mobile phases were mobile phase A, 0.1% (v/v) formic acid in deionized water, and mobile phase B, 0.1% (v/v) formic acid in methanol. Solvent flow for analysis included an initial programming of 1 min 95% mobile phase A and 5% mobile phase B, then 1 min 95% mobile phase A and 5% mobile phase B, followed by 6 min 5% mobile phase A and 95% mobile phase B, then 6 min 95% mobile phase A and 5% mobile phase B, and finally 11 min 95% mobile phase A and 5% mobile phase B. Positively charged amantadine, and IS ions were monitored using multiple reaction monitoring (MRM). Function 1 occurred between channel reactions 151.8-78.8 with collision energy between 18-30 eV. Function 2 occurred between channel reactions 180>163 with

collision energy of 14 eV. Calibrations standards ranged from 5 to 5000 ng/mL, and quality control (QC) samples were prepared at 10, 2000, and 4000 ng/mL, based on a volume of 100 μL human plasma. Blank samples were prepared and analyzed the same way as study samples, using 100 μL blank human plasma instead of test sample human plasma.

The assay was successfully qualified based on the assessment of assay specificity, selectivity, linearity, accuracy, precision, quantitation, range, recovery, and lower limit of quantitation (LLOQ) against predetermined assay acceptance criteria. Reproducibility of response ratio from 6 repeated injections was 2.8-3.8% CV, (r^2>0.99), assay accuracy ±15%, assay precision CV <9.2%, mean recovery IS 73-76% and amantadine 80-83%, and LLOQ for amantadine of 5ng/Ml.

1.2.5.2.2. Urine analysis

BRI developed a LC/MS/MS assay method for quantitation of amantadine in human urine samples. Frozen urine samples were shipped to BRI in 30 mL capped polypropylene bottles on dry ice and stored at -20°C until analyzed. After being thawed, human urine (20 μL) was added to a 1.5 mL glass HLPC vial; then 1000 μL of 0.1% (v/v) formic acid (Fisher Scientific, Ottawa, ON) in 5% (v/v) methanol/water (EMD chemicals Gibbstown, NJ) was added to the vial. The samples were spiked with 20 μL IS rimantadine HCl (10 μg/mL) (Sigma-Aldrich Canada Ltd., Oakville, ON). Then the vials were capped and vortex mixed for 10 sec and analyzed by LC/MS/MS. Samples were analyzed using an Agilent Model 1100G binary pump, Agilent Model 1100G column compartment, and Agilent Model 1100 autosampler. Sample separation was accomplished with a Synergy Hydro-RP 80A 4 μm particle size and 50 x 2.0 mm, id

column (Phenomenex, Torrence, CA), with tandem MS/MS detection using an electrospray ionization triple-quadrupole mass analyzer (Micromass Quattro®-LC triple quadrupole mass spectrometer). Column flow rate was (0.3 mL/min) and the retention times for the amantadine and rimantadine were 4.6 and 5.0 min respectively. A blank study sample was prepared in the same way using blank human urine instead of study sample urine. Calibrations standards and QC were prepared the same way as the blank human urine sample with 20 uL each of the amantadine standards or QC samples of amantadine in human urine prepared in the same way.

Calibration standards were prepared over the concentration range from 0.2 to 200 μg/mL plus blank controls, and QC samples were prepared at 0.5, 1, 100, and 160 μg/mL, based on a volume of 20 μL human urine. Standard and QC sample concentrations were corrected for the salt content of the reference standard during data analysis.

The assay results of amantadine in the urine test samples are presented in concentration of amantadine were all within the calibration range 0.2-200 ug/mL. The assay method parameters assessed including assay specificity, selectivity, linearity, accuracy, precision, quantitation range and LLOQ, were observed, and all were within the acceptance criteria described above. Reproducibility of response ratio was CV 2.8-3.8% (r^2>0.99), assay accuracy ±15%, assay precision CV <9.2%, mean recovery IS 73-76%, and amantadine 80-83%, and LLOQ for amantadine of 5 ng/mL.

1.2.6. Data Analysis

The apparent k_{el} (h^{-1}) for amantadine elimination from the plasma was calculated as the slope of the natural logarithm of the plasma amantadine concentration versus time using the computer program GraphPad Prism (version 3.0). The initial concentration (C_0) was estimated as the extrapolation of the terminal disposition rate to the Y axis (ng/mL). Plasma $t_{1/2}$ was calculated as 0.693/ $k_{el.}$ The apparent volume of distribution (V_d) was calculated as the dose of amantadine (amantadine HCl x 0.8) divided by C_0.

The plasma amantadine clearance (Cl_p (L/h)) was calculated by multiplying V_d by k_{el}. The interval renal clearance for amantadine was calculated using the area under the curve (AUC) method (Table A1.1). Renal clearance equals the amount of the drug eliminated in the urine during each time interval divided by the AUC for plasma drug concentration for the same time interval (Perrier and Gibaldi 1982). AUC was calculated with the plasma concentration data at the beginning and end of each time interval using the trapezoidal rule (Table A1.2). Creatinine clearance was calculated by multiplying urine creatinine concentration (mmol/L) by volume (mL), and then dividing the total by plasma creatinine concentration (µmol/L) multiplied by the time interval (min) over which the blood samples were collected. Renal clearance ratios of amantadine to creatinine were calculated to evaluate the effect of bicarbonate on amantadine's renal tubule secretion.

1.2.7. Statistical Analyses

Data are expressed as mean $\pm$ SD. Appropriate data were analyzed by the paired t-test using Systat for Windows, version 6.01 (Statistical Solutions Inc., Boston, MA). Remaining data were analyzed for treatment, time period, and treatment versus time period effects by mixed model repeated measures (for time) ANOVA. For the ANOVA assessments, significant differences between means were determined with Tukey's honest significant difference (HSD) test using the software program SAS, version 9.2 (SAS Institute Inc., Cary, NC) Differences between mean values with a value of $P < 0.05$ were considered to be significant.

1.3. RESULTS

Demographics of healthy volunteers

Demographics and selected serum biochemical analyses of the 12 male study volunteers who participated in the study are presented in Tables 1.1 and 1.2. The data support the good health of the study volunteers and their eligibility to participate in this study.

The pharmacokinetics of amantadine, including $t_{1/2}$, Cl_p, k_{el}, and V_d for the control arm is presented in Table 1.3. Similar data for the bicarbonate intervention arm are presented in Table 1.4. The bicarbonate intervention had significant impact on some of the pharmacokinetic parameters. The $t_{1/2}$ was increased by 97% ($P<0.001$), Cl_p decreased by 27% ($P<0.001$), k_{el} decreased by 46% ($P<0.001$), and V_d increased by 39% ($P<0.001$). Amantadine renal:plasma clearance ratio (Cl_r:Cl_p) was calculated in both study arms. Bicarbonate infusion decreased amantadine Cl_r, Cl_p, and Cl_r:Cl_p ratio (Table 1.5, Fig 1.1).

There was no treatment effect ($P=0.462$) or treatment versus time period interaction ($P=0.888$) detected for bicarbonate infusion on amantadine plasma concentrations, but there was a time period effect ($P=0.009$) that was not discriminated by the Tukey test (Table 1.6, Fig 1.2).

Table 1.1. Study volunteers demographics.

Subject	Age (yr)	Weight (kg)	Height (cm)	BMI (kg/m^2)	IBW[1] (kg)	Amantadine HCl Dose (mg)
1	46	152.3	180	46.9	75.3	200
2	26	96.6	191	23.9	61.4	200
3	27	98.6	174	32.8	69.1	200
4	29	55.7	175	18.2	70.5	200
5	29	96.1	192	26.2	85.4	170
6	28	82.4	179	25.7	74.1	200
7	34	94.3	185	27.6	79.5	190
8	30	82.4	176	26.6	71.4	200
9	26	64.0	170	22.1	65.9	200
10	29	78.3	168	27.7	64.1	200
11	30	83.5	183	24.9	77.7	200
12	28	71.0	181	21.7	75.9	200
Mean ± SD	30 ± 5	87.9 ± 23.3	179 ± 7	27.0 ± 7.2	72.5 ± 6.9	197 ± 9

BMI: Body mass index
BMI Formula = weight (kg)/height (m^2)
[1]IBW: Ideal body weight (kg)
Equation for IBW= 50 + 2.3 (ht (cm) – 152.4) / 2.54 (Devine 1974)

Table 1.2. Selected serum biochemistry data from study volunteer eligibility assessment.

Subject	Serum Urea (mmol/L)	Serum Creatinine (mmol/L)	Serum glucose (mmol/L)	Serum Sodium (mmol/L)	Serum Potassium (mmol/L)	Serum Chloride (mmol/L)
Normal range	2.8 - 7.1	44 -106	3.6 - 6.0	135-147	3.5 - 5.0	97-106
1	4.3	74	4.0	139	3.9	103
2	4.7	98	4.9	140	4.0	101
3	4.5	113	4.6	143	3.6	102
4	3.4	77	4.6	140	4.5	101
5	7.0	105	4.6	139	4.3	100
6	6.1	94	4.4	143	3.7	103
7	5.5	94	4.8	141	3.7	100
8	7.1	103	5.2	141	4.6	102
9	6.3	85	4.3	142	3.7	101
10	4.8	84	4.2	140	3.9	104
11	6.1	86	4.6	143	4.3	103
12	2.8	84	5.8	144	3.7	103
Mean ± SD	5.2 ± 1.4	91 ± 12	4.7 ± 0.5	141 ± 2	4.0 ± 0.3	102 ± 1

Summary data are presented as mean ± SD

Table 1.3. Amantadine pharmacokinetics - saline treatment arm.

Vol.	wt (kg)	Dose A-HCL (mg)	C_0 (ug/L)	k_{el}** (h^{-1})	r^2	$t_{1/2}$** (h)	V_d (L)	V_d** (L/kg)	Cl_p (L/h)	Cl_p* (mL/kg/h)	$t_{1/2}$ ratio Saline: Bicarb
1	152.3	200	365	0.029	0.92	23.1	438	2.88	12.7	83	0.51
2	96.6	200	436	0.029	0.70	24.0	367	3.80	10.6	110	0.7
3	98.6	200	572	0.066	0.96	10.5	280	2.84	18.5	187	0.75
4	55.7	170	845	0.057	0.99	12.0	161	2.89	9.2	165	0.55
5	96.1	200	523	0.066	0.98	10.5	306	3.18	20.2	210	0.58
6	82.4	200	498	0.044	0.91	15.8	321	3.90	14.1	172	0.54
7	94.3	200	337	0.055	0.93	12.5	475	5.03	26.1	277	0.35
8	82.4	200	678	0.058	0.95	11.9	236	2.86	13.7	166	0.46
9	64.0	190	692	0.046	0.87	15.2	220	3.43	10.1	158	0.75
10	78.3	200	455	0.041	0.84	16.9	352	4.49	14.4	184	0.43
11	83.5	200	545	0.066	0.99	10.5	294	3.52	19.4	232	0.58
12	71.0	200	544	0.039	0.87	17.8	294	4.14	11.5	162	0.40
Mean ± SD	87.9 ± 24.3	197 ± 9	541 ± 144	0.050± 0.014	0.91 ± 0.08	15.1 ± 4.7	312 ± 88	3.58 ± 0.71	15.0 ± 5.0	176 ± 51	0.55 ± 0.13

A : Amantadine

r^2 : Coefficient of determination

C_0: Initial concentration

* P<0.001 saline arm versus bicarbonate arm (paired t-test)

**P < 0.0001 saline arm versus bicarbonate arm (paired t-test)

Summary data are presented as mean ± SD

Table 1.4. Amantadine pharmacokinetics - bicarbonate treatment arm.

Vol.	wt (kg)	Dose A-HCL (mg)	C_0 (ug/L)	k_{el}** (h^{-1})	r^2	$t_{1/2}$** (h)	V_d (L)	V_d** (L/Kg)	Cl_p (L/h)	Cl_p* (mL/kg/h)	$t_{1/2}$ ratio Salin: Bicarb
1	152.3	200	281	0.015	0.54	45	569	3.74	8.5	56	0.51
2	96.6	200	344	0.016	0.81	43.3	465	4.81	7.4	77	0.7
3	98.6	200	437	0.049	0.90	14.0	366	3.71	17.9	182	0.75
4	55.7	170	765	0.031	0.95	22.0	178	3.19	5.5	99	0.55
5	96.1	200	308	0.038	0.68	18.0	519	5.41	19.7	205	0.58
6	82.4	200	388	0.024	0.60	29.0	412	5.00	9.9	120	0.54
7	94.3	200	224	0.019	0.31	35.5	714	7.57	13.6	144	0.35
8	82.4	200	508	0.026	0.86	26.0	315	3.82	8.2	99	0.46
9	64.0	190	528	0.034	0.95	20.4	288	4.50	9.8	153	0.75
10	78.3	200	334	0.018	0.60	39.6	479	6.12	8.6	110	0.43
11	83.5	200	372	0.038	0.84	18.2	430	5.15	16.3	196	0.58
12	71.0	200	336	0.015	0.49	45.0	476	6.71	7.1	101	0.40
Mean ± SD	87.9 ± 24.3	197 ± 9	402 ± 144	0.027 ± 0.011	0.71 ± 0.21	29.7 ± 11.5	434 ± 140	4.98 ± 1.32	11.1 ± 4.7	128 ± 48	0.55 ± 0.13

r^2: Coefficient of determination
* P<0.001 saline arm versus bicarbonate arm (paired t-test)
**P < 0.000 saline arm versus bicarbonate arm (paired t-test)
Summary data are presented as mean ± SD

Table 1.5. Amantadine renal:plasma clearance ratio (Cl_r:Cl_p) for 12 healthy male volunteers.

Saline arm			Bicarbonate arm		
Amantadine Plasma Clearance (Cl_p)	Amantadine Renal Clearance (Cl_r)	Cl_r:Cl_p*	Amantadine Plasma Clearance (Cl_p)	Amantadine Renal Clearance (Cl_r)	Cl_r:Cl_p*
mL/min	mL/min		mL/min	mL/min	
212	259	1.22	142	147	1.04
177	185	1.05	123	75	0.61
308	357	1.16	298	120	0.40
153	177	1.15	92	45	0.49
337	337	1.00	328	191	0.58
235	224	0.95	165	80	0.48
435	332	0.76	227	247	1.09
228	184	0.81	137	115	0.84
168	237	1.41	163	140	0.86
240	134	0.56	143	93	0.65
323	326	1.01	272	176	0.65
192	151	0.79	118	101	0.85
251 ± 84	242 ± 79	0.99 ± 0.23	184 ± 78	128 ± 57	0.71 ± 0.22

* (P=0.0163) amantadine Cl_r:Cl_p ratio with bicarbonate administration versus Cl_r:Cl_p ratio with saline administration (paired t-test). Summary data are presented as mean ± SD

Fig 1.1. The effect of bicarbonate versus saline infusion on amantadine renal:plasma clearance ratio (Cl_r:Cl_p) for 12 healthy male volunteers.

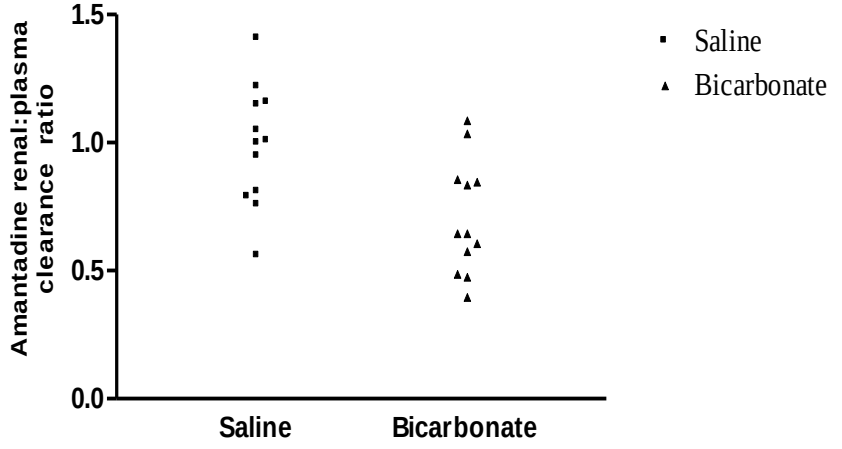
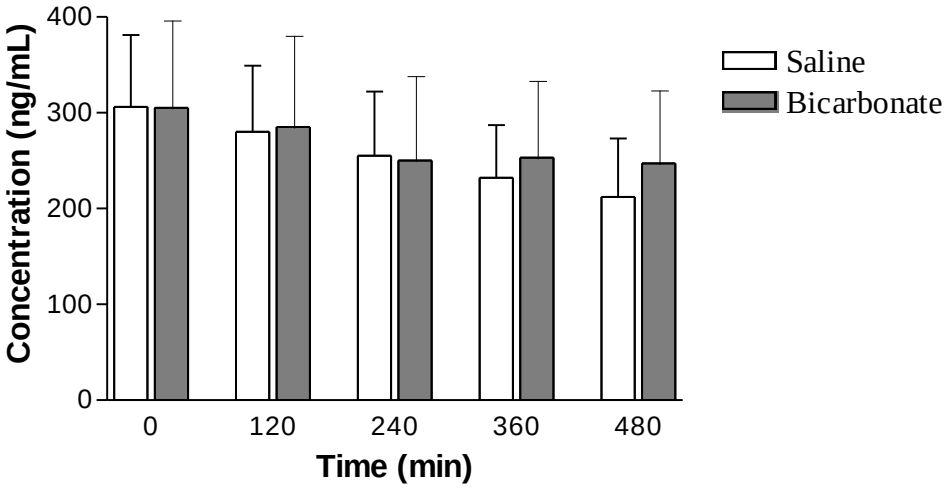


Table 1.6. Amantadine plasma concentration in different time intervals for the two study arms in 12 health male volunteers.

Time	Saline	Bicarbonate
(min)	(ng/mL)	(ng/mL)
0	306 ± 75	305 ± 92
120	280 ± 69	285 ± 96
240	255 ± 67	250 ± 89
360	232 ± 55	253 ± 81
480	212 ± 61	247 ± 77

Fig 1.2. The effect of bicarbonate versus saline infusion on mean amantadine plasma concentration on different times for the two study arms. Data are presented as mean ± SD.



The interval amantadine renal clearance (Table 1.7, Fig. 1.3A) was similar in both amantadine-treated groups before bicarbonate administration (collection period 0-2 h). Starting with bicarbonate administration (collection periods 2–8 h), the overall mean amantadine renal clearance (Cl_r) was 53-70% lower in the bicarbonate-treated group versus the amantadine plus saline treated group ($P<0.001$), Differences were detected in study day time intervals 2-4 h, 4-6 h, and 6-8 h between the two treatment arms. In the saline arm, amantadine Cl_r was decreasing with time except in the 2-4 h time interval, where amantadine Cl_r was higher than during any other study day time interval (Fig. 1.3A). With bicarbonate infusion amantadine Cl_r also was decreasing with time, except in the study day 6-8 h time interval.

There were no treatment ($P=0.157$), time period ($P=0.187$) effects, or treatment versus time period interaction ($P=0.417$) in creatinine clearance (Cl_{cr}) (Table 1.7, Fig 1.3B). Mean amantadine:creatinine clearance ratios were similar before bicarbonate treatment in both experimental arms (0-2 h study day interval), and then were decreased compared to the respective time controls after bicarbonate treatment. The overall amantadine:creatinine clearance ratio was 64-67% lower in the bicarbonate-treated arm compared with the saline-treated arm ($P<0.001$). Similar to amantadine Cl_r , the amantadine:creatinine clearance ratio decreased with time in the bicarbonate arm except in the 6-8 h study day time interval and in the saline arm was higher than any other study day time interval value only during the 2-4 h collection interval (Table 1.7, Fig 1.3C).

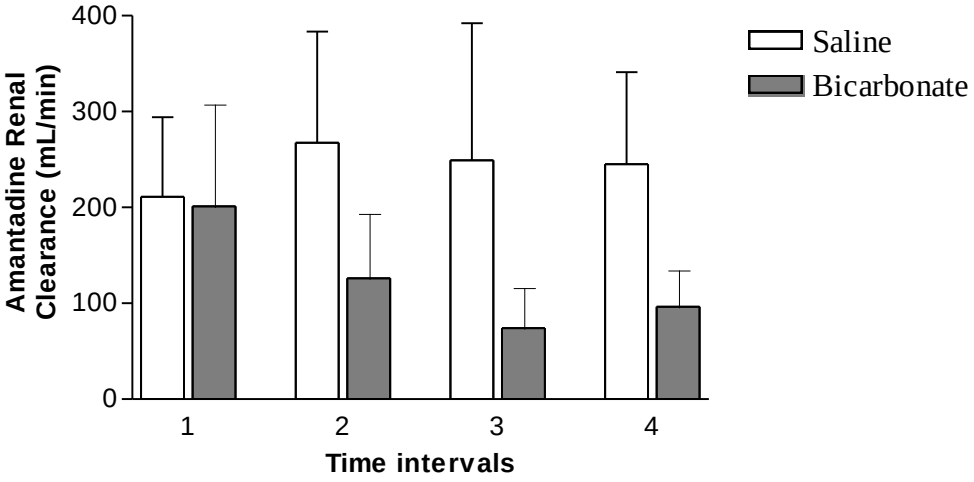
Table 1.7. Creatinine and amantadine clearance and clearance ratio. Data are presented as mean ± SD.

Interval	Treatment	0-2 h	2-4 h	4-6 h	6-8 h
Creatinine renal clearance (mL/min)	Saline	100 ± 39	122 ± 38	143 ± 62	141 ± 58
	HCO_3^-	137 ± 54	137 ± 31	133 ± 42	156 ± 50
Amantadine renal [a] clearance (mL/min)	Saline	211 ± 83	268 ± 116	249 ± 143	245 ± 96
	HCO_3^-	201 ± 107	126 ± 68	74 ± 43	96 ± 39
Clearance ratio [b] Amantadine:Creatinine	Saline	2.2 ± 0.9	2.5 ± 1.7	1.8 ± 0.6	2.1 ± 1.4
	HCO_3^-	1.5 ± 0.7	0.9 ± 0.5	0.6 ± 0.3	0.7 ± 0.3

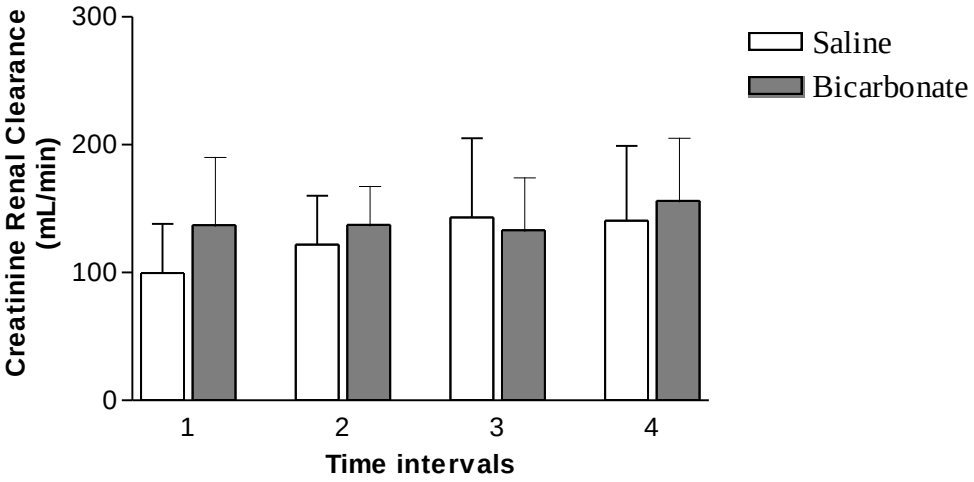
[a] There was a treatment effect ($P<0.0001$) and a treatment versus time period interaction ($P<0.019$) (ANOVA). The Tukey test showed differences between the two treatments in the 2-4 h ($P=0.014$), 4-6 h ($P=0.001$) and 6-8 h time intervals ($P=0.009$). Also there was a difference between the 0-2 h and 4-6 h time intervals ($P=0.038$) in the bicarbonate arm.
[b] There was a treatment ($P<0.0001$) and a possible time period effect ($P<0.056$) (ANOVA). The time interval difference was not discriminated by the Tukey test.
Data are presented as mean ± SD

Fig 1.3. The effect of bicarbonate versus saline infusion on mean amantadine renal clearance, creatinine renal clearance, and amantadine:creatinine clearance ratio for different time intervals on the two study arms.

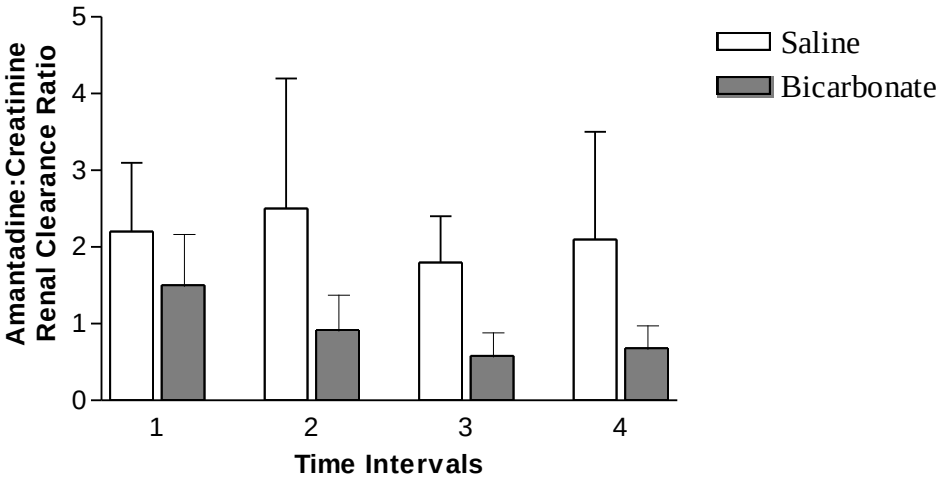
A) Amantadine renal clearance during different time intervals for the two study arms. Data are presented as mean ± SD.



B) Creatinine renal clearance during different time intervals for the two study arms. Data are presented as mean ± SD.



C) Amantadine:creatinine renal clearance ratio during different time intervals for the two study arms. Data are presented as mean ± SD.



In prestudy samples, there was no difference between the two study arms in venous blood gas values, including $HCO3^-$, pCO2, and venous blood pH. After bicarbonate infusion, there was an increase in HCO_3^- level in comparison to the control arm (P=0.0002). Also there was a time period effect (P=0.0001) and treatment versus time period interaction (P=0.001). In the bicarbonate arm, the blood HCO_3^- showed the maximum concentration during the HCO_3^- infusion interval (study day collection period 2-4 h). Beginning with the 4-6 h study day time interval, HCO_3^- blood concentration started to decrease but didn't reach the corresponding control value by the end of the 6-8 h study day time interval (Table 1.8). No difference in blood CO_2 concentration due to treatment (P=0.854) or treatment versus time period interaction (P=0.839) was observed, but there was a difference among the four collection intervals (P<0.001) that was not discriminated by the Tukey test.

In contrast to the large increase in blood bicarbonate concentration, there was a statistical difference (P<0.001) but not a clinically important difference in venous blood pH after bicarbonate administration. There was no time period effect (P=0.245), but there was a treatment versus time period interaction (P=0.0308). The only difference in pH between both arms that was detected by the Tukey test was in the study day 2-4 h time interval during which saline and HCO_3^- were infused (P<0.0103) (Table 1.8).

Table 1.8. Selected acid/base parameters for blood and urine samples for the 12 male study volunteers. Data are presented as mean ± SD.

Interval	Study arm	Prestudy	0-2 h	2-4 h	4-6 h	6-8 h
Venous Blood [a] HCO_3^- (mmol/L)	Saline	26 ± 2	27 ± 2	27 ± 2	27 ± 1	27 ± 1
	HCO_3^-	27 ± 1	28 ± 2	32 ± 3	30 ± 2	29 ± 2
Venous [b] Blood CO_2 (mm Hg)	Saline	50 ± 9	50 ± 9	58 ± 9	53 ± 8	51 ± 7
	HCO_3^-	48 ± 5	53 ± 8	58 ± 10	51 ± 9	50 ± 7
Venous Blood [c] pH	Saline	7.34 ± 0.05	7.35 ± 0.04	7.30 ± 0.04	7.33 ± 0.04	7.34 ± 0.04
	HCO_3^-	7.36 ± 0.03	7.34 ± 0.05	7.37 ± 0.05	7.39 ± 0.04	7.38 ± 0.04
Urine pH [d]	Saline	ND	6.51 ± 0.55	6.39 ± 0.53	6.02 ± 0.74	6.32 ± 0.71
	HCO_3^-	ND	6.53 ± 0.78	7.30 ± 0.33	7.66 ± 0.27	7.47 ± 0.34

ND: Not determined

[a] There was a treatment effect ($P<0.0002$), time period effect ($P<0.0001$) and treatment versus time period interaction ($P<0.001$) (ANOVA). The Tukey test showed differences between both arms in the 2-4 h time period ($P<0.0001$). In the bicarbonate arm there was a difference between prestudy and the 2-4 h time period ($P<0.0001$), prestudy and the 4-6 h time period ($P<0.009$), between the 0-2 h and the 2-4 h time periods ($P<0.0001$), between the 2-4 h and the 4-6 h time periods ($P<0.045$), and between the 2-4 h and 6-8 h time periods ($P<0.0012$).

[b] There was a time period difference ($P<0.0111$) (ANOVA), that was not discriminated by the Turkey test.

[c] There was a treatment effect ($P<0.002$), and a treatment versus time period interaction ($P<0.031$) (ANOVA). The Tukey test showed a difference in the 2-4 h time period between both arms ($P<0.0103$).

[d] There was a treatment effect ($P<0.0001$) and a treatment versus time period interaction ($P=0.0002$). The Tukey test showed a difference between both arms in the 2-4 h time period ($P=0.0073$), the 4-6 h time period ($P<0.0001$), and the 6-8 h time period ($P=0.0004$). In the bicarbonate arm the Tukey test showed a difference between the 0-2 h and the 4-6 h time periods ($P<0.0006$), and between the (0-2 h and 6-8 h time periods ($P<0.0058$).

Urine pH in the saline-treated arm remained stable and slightly acidic throughout the study day, whereas the urine pH became alkaline immediately after bicarbonate infusion ($P < 0.001$) and remained elevated to the end of the study day. The differences in urine pH between the two arms were detected in the 2-4 , 4-6 and 6-8 h study day time intervals (Table 1.8). Using the Henderson-Hasselbach equation to calculate % amantadine ionized for different blood and urine pH environments, amantadine ionization varied between (99.13% - 99.99%) in the urine and between 99.79% - 99.88 % in the blood during the study days.

No differences were detected due to treatment and treatment versus time period interaction in fluid intake ($P=0.363$, $P=0.862$) and urine output ($P=0.862$, $P=0.828$) respectively, between the two study arms, but there was a change in fluid intake and output as a function of study day time interval ($P<0.0001$) that was not discriminated by the Tukey test (Table 1.9).

In selected serum biochemistry determinations, creatinine and urea concentrations were not affected due to treatment ($P=0.711$, $P=0.320$), time period ($P=0.211$, $P=0.157$), or treatment versus time period interaction ($P=0.971$, $P=0.976$) respectively (Table 1.10). Sodium concentration also was not affected by treatment ($P=0.801$) and there was no treatment versus time period interaction ($P=0.989$). However, there was a time period effect ($P=0.0074$) that was not discriminated by the Tukey test (Table 1.10). Serum glucose concentration differed by time period ($P=0.0001$) but not by treatment ($P=0.109$) or treatment versus time period interaction ($P=0.845$).

Table 1.9. Fluid intake and urine output for 12 male volunteers. Data are presented as mean ± SD.

Interval	Study arm	0-2 h	2-4 h	4-6 h	6-8 h
Fluid intake[a] (mL)	Saline	1186 ± 436	333 ± 322	513 ± 374	468 ± 253
	HCO_3^-	1192 ± 267	374 ± 374	558 ± 254	625 ± 248
Urine output[a] (mL)	Saline	423 ± 177	831 ± 358	409 ± 222	440 ± 278
	HCO_3^-	410 ± 385	923 ± 329	546 ± 244	548 ± 298

[a] There was time period difference ($P<0.0001$) that was not discriminated by the Tukey test.

Table 1.10. Selected serum biochemistry test results for bicarbonate versus saline treatment over the 5 time periods for the twelve male volunteers. Data are presented as mean ± SD.

Interval Normal test range	Treatment arm	Prestudy	0-2 h	2-4 h	4-6 h	6-8 h
Urea 2.8-7.1 mmol/L	Saline	5.3 ± 1.5	5.1 ± 1.4	4.7 ± 1.4	4.5 ± 1.2	4.7 ± 1.2
	HCO_3^-	5.5 ± 1.2	5.4 ± 1.1	4.8 ± 1.0	5.0 ± 0.9	4.8 ± 1.1
Glucose[a] 3.6-6 mmol/L	Saline	5.2 ± 0.7	4.6 ± 0.7	5.1 ± 1.0	4.1 ± 0.6	4.8 ± 0.6
	HCO_3^-	5.4 ± 0.8	4.8 ± 0.9	5.5 ± 0.7	4.5 ± 1.0	4.7 ± 0.9
Sodium[b] 135-147 mmol/L	Saline	140 ± 3	139 ± 3	141 ± 2	142 ± 2	142 ± 2
	HCO_3^-	141 ± 3	140 ± 2	141 ± 2	142 ± 3	142 ± 2
Potassium[c] 3.5-5.3 mmol/L	Saline	3.9 ± 0.3	4.1 ± 0.4	3.9 ± 0.3	4.0 ± 0.4	4.1 ± 0.3
	HCO_3^-	3.9 ± 0.5	4.0 ± 0.4	3.6 ± 0.2	3.7 ± 0.3	3.7 ± 0.3
Chloride[d] 97-106 mmol/L	Saline	103 ± 2	101 ± 1	104 ± 1	104 ± 2	104 ± 1
	HCO_3^-	103 ± 2	102 ± 3	100 ± 2	102 ± 5	101 ± 2
Creatinine 44-106 μmol/L	Saline	94 ± 15	88 ± 12	83 ± 13	86 ± 11	87 ± 15
	HCO_3^-	90 ± 12	89 ± 12	84 ± 11	84 ± 15	87 ± 12

[a] There was time period difference ($P<0.0001$) (ANOVA). The Tukey test showed difference in the saline arm between period 0-2 h and 6-8 h ($P=0.0523$).

[b] There was time period difference (P<0.0074) (ANOVA), that was not discriminated by the Tukey test.
[c] There were treatment (P<0.0161), and time period (P<0.0491) effects that were not discriminated by the Tukey test.
[d] There was a treatment effect (P<0.001), and treatment versus time period interaction (P 0.0021). The Tukey test showed a difference between prestudy and the 0-2 h time period (P<0.0191) in the bicarbonate arm and in the 2-4 h time period between both arms (P=0.0003).

Chloride ion concentration was statistically higher in the saline study arm (P=0.001), but this difference was not clinically important. There was a treatment versus time period interaction (P=0.0021) but no difference detected due to study day time interval (P=0.136). In the study day 2-4 h time interval, during which saline and bicarbonate was administered, serum Cl^- concentration was higher in the saline arm and lower in the bicarbonate arm (P=0.0003).

Potassium concentration was slightly decreased (P=0.0161) in the bicarbonate arm compared with the saline arm. This difference didn't exceed the K^+ physiological normal range. There was a time period effect (P=0.049) not discriminated by the Tukey test, but no treatment versus time period interaction was detected (P=0.207) (Table 1.10). None of the acid-base parameters or selected serum biochemistry values exceeded the normal ranges provided by the Hospital Clinical Biochemistry laboratory.

1.4. DISCUSSION

This study addressed in humans the previously described amantadine bicarbonate-dependent renal tubule transport mechanism *in vitro* and *in vivo* in rats (Escobar, Wong et al. 1994; Goralski, Smyth et al. 1999). It was demonstrated that an acute bicarbonate load administered to healthy male volunteers impairs amantadine renal secretion without a change in serum creatinine concentration or urinary creatinine clearance.

In 1967, Geuens et al conducted a study on 3 healthy volunteers over a period of 14 days during which 650 mg of amantadine was administered (150 mg priming dose then 50 mg daily for 10 days). From the 6^{th} day sodium bicarbonate was administered (150 mEq /day) divided into three doses of 4.2 g as an effervescing drink containing an undeclared amount of citric acid. After the administration of sodium bicarbonate, the body content of amantadine rose steadily. When the experiment was stopped, there was a residual body burden of about 350% of the daily dose. This decrease in amantadine renal clearance was explained as a pH mediated passive reabsorption, and the possibility of bicarbonate ion driven changes in secretion or filtration was not considered.

In 1997, a three arm study was conducted in 15 pentobarbital-anaesthetized male dogs in which amantadine and creatinine renal clearance were determined after an i.v. dose of 3mg/kg amantadine HCl (arms 1 -3), and i.v. sodium bicarbonate (arm 2) or sodium lactate (arm 3) loading dose (5 mmol/kg) followed by a maintenance infusion of bicarbonate or lactate to maintain stable venous concentrations. Infusion of sodium lactate or sodium bicarbonate began 2 h after the amantadine dose, and there was a terminal 2 h collection period at the end of their infusion (6-8 h after the start of the

amantadine dose). The control amantadine Cl_r (arm 1) was (11.1 ±1.6 mL/kg/min) and decreased to (1.9 ± 0.4 mL/kg/min) after sodium bicarbonate infusion. Thus amantadine Cl_r decreased by 83% (Sitar, Escobar et al. 1997). In our human study, amantadine Cl_r was decreased by 53-70%.

Neither of the previous two studies excluded the known action of Na^+ on aldosterone and angiotensin that affect the renal tubular control of organic ion excretion. Sodium dependent efflux of organic cations has been shown for the luminal membrane, in which the Na^+/H^+ exchanger is present (Holohan and Ross 1981; Katsura, Ekholm et al. 1991). In 1999,

A rat study by Goralski et al investigated the effect of bicarbonate on renal Cl_r and urinary excretion of amantadine (organic base) and kynurenic acid (organic acid). After a moderate diuresis was established, animals received i.v. (3mg/kg) amantadine HCl or kynurenic acid followed by an acute dose of sodium bicarbonate (2000 mOsM/L, 5 mmol/kg i.v. at 111 µL/min) or physiological saline (79 µL/min) to provide an equivalent body burden of Na^+. Urine and blood samples were collected for five successive 20 min collection periods and analyzed for amantadine or kynurenic acid, blood gases, and pH. In that study the interval amantadine Cl_r was 30-60% lower after bicarbonate administration than for the respective control. In our study the reduction the amantadine renal clearance was 53-70%.

In our study an i.v. infusion of 2 mmol/kg isotonic sodium chloride solution served as a control for the sodium load resulting from the hypertonic sodium bicarbonate infusion (8.4% USP; 1 mmol/mL solution) administered over a 2 h time interval from 1000 to 1200 h. The i.v. route insured complete absorption of Na^+ and HCO_3^-, and

eliminated the potential for nausea and vomiting associated with oral ingestion of a large volume of salty solution. This dose of sodium bicarbonate is safe in humans (Isozaki, Kumagai et al. 1995; Nakashima, Yamashita et al. 1996).

In the control arm of the present study, the pharmacokinetics of amantadine were consistent with the normal values reported in the literature (Aoki and Sitar 1988). After the bicarbonate administration, the pharmacokinetic parameters and amantadine clearance were affected significantly. The acute dose of bicarbonate was sufficient to impair the Cl_r of amantadine for an extended period of time. It was important to compare the interval Cl_r rather than just the overall renal clearances so the persistence of any effects of bicarbonate treatment could be identified.

Creatinine clearance was used as a general marker for renal glomerular filtration (Fu, Hsu et al. 1959). The contribution of amantadine secretion to its overall renal clearance was evaluated by the amantadine:creatinine clearance ratio. Amantadine undergoes significant renal tubule secretion, as indicated by an amantadine:creatinine clearance ratio of more than 1 (Table 7 and fig 2A). In our study amantadine:creatinine clearance ratios were 64-67% lower than control after bicarbonate treatment compared to 55-70% lower than the respective time controls in the rat study (Goralski, Smyth et al. 1999). Based on the decrease in the observed amantadine:creatinine clearance ratio in face of unchanged creatinine clearance, we conclude that bicarbonate administration decreased amantadine clearance through effects on secretion and not on filtration. The mean amantadine Cl_r:Cl_p ratio (0.99 in control, and 0.71 in the bicarbonate-treated arm) are consistent with routes of amantadine renal elimination in addition to renal excretion after administration of bicarbonate.

We assume that this effect of sodium bicarbonate administration on amantadine clearance is the result of high bicarbonate concentration observed right after sodium bicarbonate infusion. Venous bicarbonate concentration was 4 mmol/L higher than the respective control and remained elevated to the end of the study. In contrast, venous blood pH was increased only statistically, but not to a clinically important degree after bicarbonate administration. Furthermore pCO_2 level was not changed between the two study arms, suggesting that no compensation due to an increase in blood pH had occurred.

We believe that Na^+ has no or little effect on the depression of amantadine renal clearance, because there was no difference in sodium level between the control and the study arm and the Na^+ level remained within the normal range for both arms throughout the study. In addition, *in vitro* studies have suggested that amantadine transport into isolated renal proximal and distal tubules is independent of Na^+ concentration in the incubation medium. However requirement of Na^+ cannot be ruled out as extracellular Na^+ was not abolished completely or was intracellular sodium manipulated directly (Escobar and Sitar 1996). Also passive reabsorption might have only minor role in explaining the decrease in amantadine clearance because:

1- The high p*K*a of amantadine (p*K*a 10.1), and high percent of amantadine ionized in different blood and urine pH during study periods varied between (99.13% - 99.99%) which will allow only limited gradient for passive reabsorption of amantadine from the tubule lumen to the peritubular capillaries.

2- With increasing urine flow rates with time, there would be a predicted increase in net renal drug excretion and increased clearance due to less contact time for

passive reabsorption of the drug into the capillaries. This effect was not apparent because there was no difference between fluid intake and fluid output between the two study arms. Similar effects and results were reported in uninephrectomized rat experiments studying the effect of bicarbonate on renal tubule uptake of amantadine (Goralski, Smyth et al. 1999).

The levels of serum urea and creatinine were not different between the two treatments arms, which suggested normal renal function throughout the study. Sodium chloride and sodium bicarbonate both were administered in 5% wv glucose in water solution; so there was no difference between two treatment arms in serum glucose concentration. Serum chloride ion concentration was slightly higher in the saline arm ($P<0.001$) but this increase was not clinically important as chloride level remained within the normal range. The slight increase in Cl^- concentration in the saline arm might be explained by the administration of Cl^- ion in NaCl solution. Serum K^+ decreased after bicarbonate infusion, which was expected, as bicarbonate ion will stimulate an exchange of cellular H^+ for Na^+, thus leading to stimulation of the sodium-potassium ATPase, which will shift K^+ from extracellular to intracellular (Gutierrez, Schlessinger et al. 1991).

The exact mechanism of the bicarbonate-mediated decrease in amantadine clearance was not determined. The identity of the amantadine transporters remains elusive (Goralski, Lou et al. 2002; Wright, Evans et al. 2004). Similar effects of bicarbonate loading on decreasing the renal clearance of other organic bases in rats and dogs have also been reported (Torretti, Weiner et al. 1962; Roch-Ramel, Diezi-Chomety et al. 1980). Some evidence exists that indicates secretion of some organic cations across

the brush border membrane of proximal tubules is coupled to an inwardly directed proton gradient that is driven by the Na^+/H^+ exchanger located in the brush border membrane (Holohan and Ross 1981; Takano, Inui et al. 1984; Rafizadeh, Roch-Ramel et al. 1987). Therefore, it is possible that the alkalinization of the tubule fluid that occurs after bicarbonate administration may cause a decrease in the driving force for H^+/organic cation exchange across the brush border membrane of proximal tubules and thus a decrease in amantadine clearance (Goralski and Sitar 1999). It was reported that amantadine and TEA have distinct basolateral transport mechanism in renal tubules (Goralski and Sitar 1999).

Transport sites in the proximal tubule can be subdivided into bicarbonate-dependant (high affinity, high capacity) and less efficient bicarbonate-independent (low affinity, low capacity) types (Escobar, Wong et al. 1994; Escobar and Sitar 1995). It was hypothesized that amantadine uptake via rOCT1 and rOCT2 is minimal compared with TEA, and that rat renal proximal tubules contain a novel type of OCT in addition to rOCT1 and rOCT2 that mediates amantadine uptake and requires bicarbonate for optimal function (Goralski, Lou et al. 2002).

Other studies directed toward understanding the mechanism behind an increase in amantadine transport in kidney tissue from diabetic rats (Goralski, Stupack et al. 2001; Grover and Atwal 2002) reported that regulation of amantadine transport was mediated by soluble adenylyl cyclase, suggesting that cAMP may be important in determining overall cellular transport for organic cations. Soluble adenylyl cyclase activity is known to be modulated by bicarbonate and lactate, which might explain the bicarbonate effect on amantadine transport (Gerlyand and Sitar 2009).

In summary our study supports a previous report that chronic administration of bicarbonate reduced the renal excretion of amantadine in humans (Geuens and Stephens 1967). However, we demonstrated that this reduction was not due to the change in pH. Our study and the previous *in vitro* and *in vivo* rat studies (Escobar, Wong et al. 1994; Goralski, Smyth et al. 1999) demonstrated bicarbonate-dependent organic cation transport which further suggests that bicarbonate may modulate specific renal tubule organic cation transporters, which impair organic cation secretion, as a mechanism by which bicarbonate administration decreases organic cation renal clearance as represented by amantadine clearance in our study.

Some organic cationic drugs are highly toxic to the kidney (Bennett 1989). Change in acid/base status due to chronic or acute illness, or aging might result in increased plasma bicarbonate levels that compromise renal elimination of amantadine and possibly other organic cation drugs that are specifically handled by bicarbonate-dependent organic cation transporters in the kidney, causing alteration in drug disposition that might cause change in efficacy and safety. The mechanism of renal organic cationic transporters regulations remains incompletely understood and further studies will be necessary for better understanding of both transporter regulation and subsequent consequences for cationic drug elimination by the kidney.

2. CHAPTER 2: DEVELOPMENT OF AN *IN VIVO* RAT MODEL FOR INDUCTION OF SPERMIDINE/SPERMINE N^1-ACETYLTRANSFERASE

2.1. INTRODUCTION

Amantadine (1-aminoadamantane) is commonly used for the control of tremor in Parkinson's disease or as antiviral in the treatment and prevention of type A influenza infection. Amantadine is an organic cationic drug that is eliminated from the body primarily by the kidneys, and renal tubule secretion is an important mechanism (Wu, Ing et al. 1982). An early report demonstrated that one aspect of amantadine disposition involved its acetylation (Koppel and Tenczer 1985) .

It was thought that arylamine *N*-acetyltransferases (NAT1, NAT2), which were considered responsible for the acetylation of many primary amino-containing drugs, were also responsible for acetylation of amantadine. NATs are phase II xenobiotic metabolism enzymes that catalyze the detoxification of arylamines (Hein, McQueen et al. 2000). While conducting preliminary studies in our laboratory on the mechanism of amantadine acetylation, it was determined that amantadine is not acetylated by either NAT1 or NAT2 (Bras, Hoff et al. 1998). A novel pathway, catalyzed by spermidine/spermine N^1-acetyltransferase (SSAT1), an enzyme not normally considered to be a catalyst for acetylation of drugs containing a primary amino group, was identified as the mechanism for amantadine acetylation (Bras, Janne et al. 2001).

Further study using transgenic mice with enhanced SSAT1 activity enabled our laboratory to demonstrate the ability of this enzyme to produce the metabolite

N-acetylamantadine (ACA) and the ability of amantadine to competitively inhibit spermidine acetylation by SSAT1 *in vitro* (Bras, Janne et al. 2001).

Amantadine is a specific substrate for SSAT1 which is a key enzyme in maintaining polyamine homeostasis and influencing cellular processes, such as normal and neoplastic growth, that are related to polyamine content. Recently, other potential functions of SSAT1, including roles in obesity/glucose tolerance, integrin function, stress response, and oxygen homeostasis have been proposed. Also alterations in SSAT1 have been linked to a variety of pathological conditions (Pegg 2008). Patients with cancer have enhanced polyamine metabolism, enhanced acetylation of many primary amino-containing drugs, and an induced level of SSAT1 in tumor tissue (Butcher et al, 2007). These biological observations suggest that a potential treatment strategy is to increase polyamine catabolism by further inducing SSAT1 activity to cause a reduction in polyamine pools as a mechanism to trigger an apoptotic response in the tumor. The resulting effect of SSAT1 induction on acetylation of concurrently administered drugs containing primary amino groups is unknown.

SSAT1 is highly regulated. Normally its intracellular level is low, but it is a stress-induced protein, and this effect has been demonstrated by many factors, such as heat shock and chemical stress (Matsui, Otani et al. 1983; Fuller, Carper et al. 1990). SSAT1 can be rapidly induced by elevating intracellular polyamine concentrations or by treating cells with a polyamine-mimetic. Seiler has identified other SSAT1 inducers in the rat, including methylglyoxal bis(guanylhydrazone), thioacetamide, carbon tetrachloride, spermidine and spermine (Seiler 1987). N-terminal substituted polyamine analogues are not substrates for SSAT1, but appear to mimic the endogenous polyamine

and cause an increase in intracellular SSAT1 activity that can be 1000-fold higher than that in untreated cells (Libby, Ganis et al. 1991). N^1, N^{11}-diethylnorspermine (DENSPM), is a candidate drug which can be used to induce SSAT1.

DENSPM is a potent SSAT1 inducer and has been extensively studied *in vivo* and *in vitro* in humans and animals. Preclinical toxicological studies were carried out in rats and dogs and the safe dose of DENSPM was determined using multiple different once daily doses for 5 days by intravenous (i.v.) injection. The doses were 100, 50, 25, and 12.5 mg/kg. Rats that received 12.5, 25, or 50 mg/kg tolerated treatment well and didn't display any physical signs that were considered abnormal (Kanter, Bullard et al. 1994). It is currently under investigation as an anti-cancer agent (Hector, Tummala et al. 2008).

Ethanol (Alc) is another interesting agent that is known to increase the intracellular level of SSAT1 (Casero and Pegg 1993; Perin and Sessa 1993). It has been appreciated for a long time that Alc ingestion is associated with an increase in isoniazid acetylation in both humans and in rats (Lester 1964; Thomas and Solomonraj 1977). It was reported that slow and fast acetylators excreted increased fractions of administered doses of sulfadimidine and procainamide as the respective acetyl metabolites when humans ingested Alc concurrently (Olsen and Morland 1978; Olsen and Morland 1982). In an *in vitro* study using suspensions of isolated rat liver parenchymal cells, the effect of Alc on acetylation of sulfanilamide and procainamide was studied. It was found that Alc treatment enhanced sulfanilamide acetylation, whereas the acetylation of procainamide was unchanged (Olsen and Morland 1983). Although Alc is an inducer of acetylation by SSAT1, the effect is not specific for this enzyme. It is known that Alc has

effects on other enzymes in human liver. After chronic Alc consumption, the activity of the microsomal ethanol-oxidizing system (MEOS) increases, with an associated rise in cytochromes P-450, especially the isoform CYP2E1. This induction is associated with proliferation of the endoplasmic reticulum, both in experimental animals and in humans, which affects the metabolism of other xenobiotics (Lieber 1999; Lieber 2004).

In our pilot study, we tried to develop a cheaper, practical model to induce SSAT1, which would allow us to further investigate if SSAT1 is a functionally important drug metabolizing enzyme in the acetylation of substrates other than amantadine, or whether amantadine is unique in its ability to be acetylated only by SSAT1. A transgenic mouse model overexpressing SSAT1 has been developed, but it is expensive. In addition it has a striking phenotype characterized by substantial alteration of polyamines pools, hair loss, female infertility, weight loss and altered lipid metabolism (Pegg, Feith et al. 2003). Alc is an appealing agent to test because it is commonly used socially by humans, easy to obtain, cheaper than other specific liquid formulae, and safe when administered to rats in drinking water. It was reported that rats fed Alc in drinking water did not develop liver lesions unless they were not eating enough food (Lieber 2004). DENSPM is a known potent SSAT1 inducer and its safe dose was established (Kanter, Bullard et al. 1994).

Our first hypothesis was that chronic exposure to Alc in drinking water will increase SSAT1 in the rat liver, and that this increased activity will be reflected by the presence of ACA in rat urine. Our second hypothesis proposed that chronic exposure to DENSPM, known to increase SSAT1 in rat liver, will be reflected by the increased presence of ACA in rat urine. Finally our third hypothesis was that combined exposure

of rats to DENSPM and Alc will result in a greater increase of rat liver SSAT1 than produced by the individual substances. The synergistic effect will be detected by the increased presence of ACA in rat urine.

2.2. EXPERIMENTAL METHODS

2.2.1. Animals

The experiments were conducted on male Sprague-Dawley rats (250-300g) (Charles River, Canada, breeding stock, University of Manitoba, Winnipeg, Manitoba) and were approved by the Bannatyne Campus Protocol Management and Review Committee at the University of Manitoba (#06-007). For the duration of the experimental procedures, rats were allowed free access to rat chow pellets and fluids as described below. Alc solutions were made by dissolving the required volume of 95% v/v Alc in sufficient distilled water to result in drinking solutions containing 0%, 5%, 10% or 15% v/v Alc in water. Animals were housed in a room with temperature control between 22 - 24 °C and with light from (0700 – 1900 h) and dark from (1900 – 0700 h).

2.2.2. Injection Solutions

The stock amantadine HCl for injection was prepared by dissolving amantadine HCl (Sigma-Aldrich Canada, Ltd., Oakville, ON) in normal saline for injection (Baxter Corp., Mississauga, ON) (3.0 mg/mL). The solution was then filtered into a sterile vial using a 0.22μm GV Millex filter (Millipore Canada, Mississauga, ON), sealed, and stored at 4°C. The DENSPM solution was prepared by dissolving DENSPM powder (provided by Genzyme Corp, Cambridge, MA, USA) in sterile water (4.3mg/mL), by injecting distilled water through a 0.22 μm GV Millex filter and directly through the rubber closure cap of the sterile vial. All animal injections were completed using sterile 1.0 mL tuberculin syringes (Baxter Corp., Mississauga, ON).

2.2.3. First Experiment

Control: Two rats received intraperitoneal (i.p.) injections with 3.0 mg/kg amantadine HCl (3.0 mg/mL) at about 10:00 am. They were placed in separate metabolic cages, and total urine was collected from the metabolic cages between 0-6 and 6-24 h.

Intervention: The rats were exposed to Alc in their drinking water at 5%, 10%, and 15% v/v respectively for one week at each concentration. At the end of every week, rats received i.p. injections with 3.0 mg/kg amantadine HCl (3.0 mg/mL). The total urine was collected as described above for the control study.

Post-Intervention Control: Subsequently, the rats were allowed to drink water for one week, and amantadine HCl was injected and urine was collected as before.

Urine samples: The volume and the pH of all urine samples were measured; then the samples were frozen and stored at -20°C until analyzed for ACA. Further details about the study are presented in the Appendix, Tables A2.1 and 2.2. After the post-intervention control study, rats were euthanized with i.p. pentobarbital sodium injection (65mg/kg). To insure death, pentobarbital-euthanized rats were subject to a midline abdominal incision and sectioning of the aorta using a scalpel and surgical scissors.

2.2.4. Second Experiment

Intervention: Two rats received i.p. injections with a dose of 50 mg/kg DENSPM for 5 days. On the 5^{th} day amantadine HCl solution (3.0 mg/kg) was injected i.p. 1 h after the DENSPM dose. The rats were placed in separate metabolic cages. Total urine was collected over 0-6 and 6-24 h after the amantadine HCl dose as described above. Further details about the study are presented in the Appendix, Tables A2.3 and 2.4.

Post-Intervention Control: Subsequently, the rats were allowed to drink water for one week, and amantadine HCl solution was injected i.p. and urine was collected as before. The volume and the pH of all urine samples were measured; then the samples were frozen and stored at -20°C until analyzed for ACA. After the post-intervention control study, rats were euthanized with i.p. pentobarbital sodium injection as described above.

2.2.5. Third Experiment

Intervention: Two rats were placed in separate metabolic cages. They were exposed to 5%, 10%, and 15% v/v Alc in drinking water respectively for 2 days each. Then the rats were exposed to both 15% v/v Alc and i.p. DENSPM injections (50mg/kg) for an additional 5 days. Amantadine HCl solution (3.0 mg/kg) was injected i.p. 1 h after the 5^{th} dose of DENSPM. Total urine was collected and recorded as described above for previous studies.

The volume and the pH of all urine samples were measured; then the samples were frozen and stored at -20°C until analyzed for ACA. Further details about the study are presented in the Appendix, Tables A2.5 and A2.6. Rats were euthanized with i.p. pentobarbital sodium injection as described above.

2.2.5. Fourth Experiment:

Intervention: Two rats were placed in separate metabolic cages. They received i.p. injections with a dose of 50 mg/kg DENSPM for 5 days. On the 5^{th} day, amantadine HCl solution (3.0 mg/kg) was injected 1 h after the DENSPM dose. Total urine was collected and documented as described above.

Post-Intervention Control: Subsequently, the rats were allowed to drink water for one week, and amantadine HCl was injected and urine was collected as before.

The volume and the pH of all urine samples were measured; then the samples were frozen at -20°C until analyzed for ACA. Further details about the study are presented in the Appendix, Tables A2.7 and A2.8. After the post-intervention control study, rats were euthanized with i.p. pentobarbital sodium injection as described above.

2.2.5. Fifth Experiment:

Intervention: Two rats were exposed to Alc in their drinking water at 5%, 10%, and 15% v/v respectively for one week at each concentration. At the end of every week, rats were injected i.p. with 3.0 mg/kg amantadine HCl (3mg/mL). Then rats were exposed to both 15% v/v Alc and DENSPM injections (50mg/kg) for another 5 days and administered amantadine HCl solution i.p. (3.0 mg/kg) 1 h after the last DENSPM dose. The total urine was collected as described before. Further details about the study are presented in the Appendix, Tables A2.9 and A2.10.

Post-Intervention Control: Subsequently, the rats were allowed to drink water for one week, and amantadine was injected and urine was collected as before. After the post-intervention control study, rats were euthanized with i.p. pentobarbital sodium injection as described above.

2.2.6. Analytical method for N-acetylamantadine (ACA) in rat urine

Initial analysis occurred on selected urine specimens by our previously described gas-liquid chromatographic method (GLC) (Bras et al., 1998). A newly developed high performance liquid chromatograph/mass spectrometry (LC/MS/MS) assay became available after the initiation of the present studies. Remaining urine specimens were analyzed using this new analytical approach that is 10 – 100 *x* more sensitive than the GLC assay. We have presented only data from samples that were able to be quantified by the LC/MS/MS technique.

2.2.6.1. High performance liquid chromatography/Mass spectrometry (LC/MS/MS) method

The samples were analyzed using a qualified method initially developed by Biopharmaceutical Research Inc. (BRI) for measurement of ACA and amantadine in human urine. Calibration standards were prepared over the concentration range from 0.1-100 ng/mL for ACA plus blank controls based on a 1000 µL rat urine sample. The blank rat urine was obtained from Sprague-Dawley rats (Bioreclamation Inc., New York, NY). Quality control (QC) samples in rat urine were prepared at 0.4, 4, 20, and 80 ng/mL for ACA. All calibration standards, QC samples, and test samples were spiked with the internal standard (IS) N-acetyl-d3-amantadine (HPSI, Mississauga, ON), and processed by liquid-liquid extraction. In a 16 x 100 mm screw-capped test tube 20 µL lS (2.0 µg/ml) were added; then 20 µL of an appropriately prepared ACA working solution and 20 µL of an appropriately prepared amantadine HCl stock solution were added to the above tube; 1000 µL blank rat urine was added followed by vortex mixing; 500 µL 1M phosphate buffer (pH 7.4) (Fisher Scientific, Ottawa, ON) was added to the above

tubes followed by vortex mixing; 5mL ethyl acetate (EMD Chemicals Inc., Gibbstown, NJ) also was added to above tubes followed by vortex mixing for 1 min and centrifugation at 3270 x g for 5 min. The supernatant was transferred to clean 13 x 100 mm tubes and evaporated to dryness at 37^{o}C in a water bath. The dried residue was reconstituted in 200 µL of 50% methanol (EMD Chemicals Inc., Gibbstown, NJ) containing 0.05% v/v formic acid (Fisher Scientific, Ottawa, ON) by vortex mixing for 1 min and sonicating at 25^{o}C for 2 min. The sample was transferred to a 250 µL HPLC vial, centrifuged at 3270 x g for 5 min and then analyzed by HPLC, with an Agilent Model 1100G binary pump, Agilent Model 1100G column compartment, and Agilent Model 1100G autosampler (Mississauga, ON). Sample separation was accomplished with a Synergy Hydro-RP 80A 4 µm particle size and (50 x 2.0 mm, id) column (Phenomenex, Torrence, CA), with tandem MS/MS detection using an electrospray ionization triple-quadrupole mass analyzer (Micromass Quattro®-LC triple quadrupole mass spectrometer; Micromass Canada, Pointe-claire, QC). HPLC had two mobile phases, mobile phase A (0.1% v/v formic acid in deionized water) and mobile phase B (0.1% v/v formic acid in methanol). Solvent flow for analysis includes an initial condition after sample injection of 1 min with 95% mobile phase A and 5% mobile phase B, then gradient change to 1.1 min to result in a solvent composition of 5% mobile phase A and 95% mobile phase B that was maintained until 6 min. The gradient was then reversed to 95% solvent A and 5% solvent B until 6.1 min. This solvent composition was maintained until 11 min, when the next sample was injected for analysis. Positively charged ACA and IS ions were monitored using multiple reaction monitoring (MRM) mode. Quantitation of ACA in rat urine was performed on the peak

area response ratio of ACA (channel reaction between 193.9-92.8 with collision energy between 18-28 eV) and to the IS (channel reaction 196.8-92.8 with collision energy between 18-32 eV) spiked at a constant level to all samples. Column flow rate was (0.3 ml/min) and the retention time for ACA and IS was 5.8 min. ACA and IS were differentiated through different masses, the IS having a molecular weight three Daltons greater than ACA produced by the rats.

All standards and QC samples of ACA passed the assay acceptance criteria. Reproducibility of response ratio was of 4.7% coefficient of variation (CV) for ACA/IS, (r^2>0.99). Assay accuracy was ± 15%, and assay precision was within 12% (CV) at each QC level tested for ACA. The peak response of the IS was 11% of ACA. The lower limit of quantitation (LLOQ), and peak response of low concentrations of ACA were not more than 0.06% of that of IS, and the LLOQ was 0.1 ng/mL. The volume of three experimental urine samples was less than the 1.0 ml the volume specified for the assay. For these specimens, the maximum sample volume available for the analysis was recorded and the concentration reported was corrected accordingly.

2.2.7. Data Analysis

Given the preliminary nature of this model development and the minimal number of animals authorized by the Bannatyne Campus Protocol Management and Review Committee at the University of Manitoba, no formal statistical analysis of these data were completed. The findings are described in general terms to inform the design of more thorough experiments that will define the final model that will be used to screen other candidate molecules for acetylation by SSAT1. Where there was sufficient replication of a particular experimental intervention, data are presented as mean ± S.D.

2.3. RESULTS

In the first experiment, the control production of ACA was absent or present only in trace amount (0-5 µg) in rat urine after either 0-6 or 6-24 h urine collections (Fig. 2.1 and Table 2.1). At the end of every week of Alc consumption in drinking water, the amount of ACA in the 6 - 24 h urine collection was greater than in the 0 - 6 h sample. The amount of ACA production increased with increasing doses of Alc. However, the rate of ACA produced/h is greater in the 0-6 h sample. The post intervention control experiment showed that after a one week washout period there remained a detectable amount of ACA in the 0 - 6 h samples (16 µg, 16.5 µg), but a trace or non-detectable amount in the 6 - 24 h samples (0, 3.2 µg).

In the 2nd experiment we were unable to reanalyze the urine samples using the LC/MS/MS assay due to an insufficient residual urine sample. Only trace amounts were detected at the post intervention control 6-24 hr (3.34 µg, 2.53 µg) (Fig. 2.2 and Table 2.2). The 4th experiment includes the same protocol for inducing SSAT1 activity by DENSPM injection, and is interpreted together with the present protocol. The 4th experiment showed an increase in ACA after DENSPM administration. The amount of ACA produced and the rate of its production in the 0 - 6 h specimen was greater than in the 6 - 24 h specimen, in contrast to our observations in experiment. The post intervention control showed (either no detectable or a trace amount of ACA in the urine (Fig. 2.4 and Table 2.4).

Figure 2.1. Acetylamantadine (ACA) excreted (µg, 0-24 h urine) vs. treatment after amantadine HCl (3mg/kg).

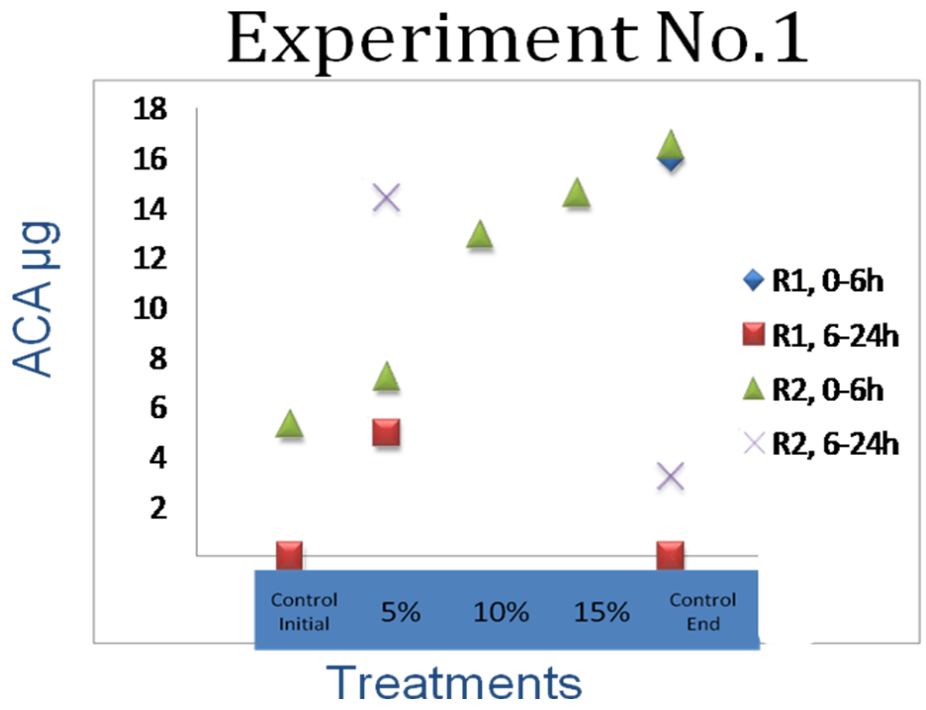


Table 2.1. Numerical data for Figure 1 presented above. Data as total metabolite (ACA) excretion in µg after amantadine HCl (3.0 mg/kg).

Rat (time interval)	Control initial	5% Alc	10% Alc	15% Alc	Control end	Rat (time interval)
R1, 0-6h					16	R1, 0-6h
R1, 6-24h	0	4.96			0	R1, 6-24h
R2, 0-6h	5.3	7.2	12.92	14.6	16.53	R2, 0-6h
R2, 6-24h		14.4			3.2	R2, 6-24h

R1: Rat with ear punch in experiment 1
R2: Rat without ear punch in experiment 1

Figure 2.2. Acetylamantadine (ACA) excreted (µg, 0-24 h urine) vs. treatment after amantadine HCl (3.0 mg/kg).

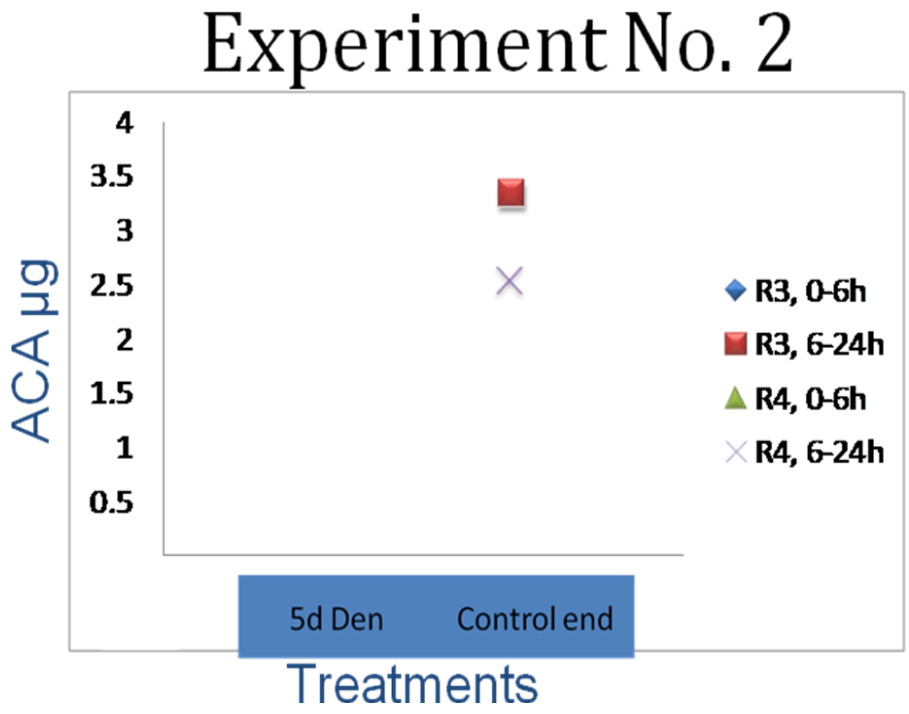


Table 2.2. Numerical data for Figure 2 presented above. Data as total metabolite (ACA) excretion in µg.

Rat (time interval)	DENSPM	Control end
R3, 0-6h		
R3, 6-24h		3.34
R4, 0-6h		
R4, 6-24h		2.53

R3: Rat with ear punch in experiment 2
R4: Rat without ear punch in experiment 2

Figure 2.3. Acetylamantadine (ACA) excreted (µg, 0-24 h urine) vs. treatment after amantadine HCl (3.0 mg/kg).

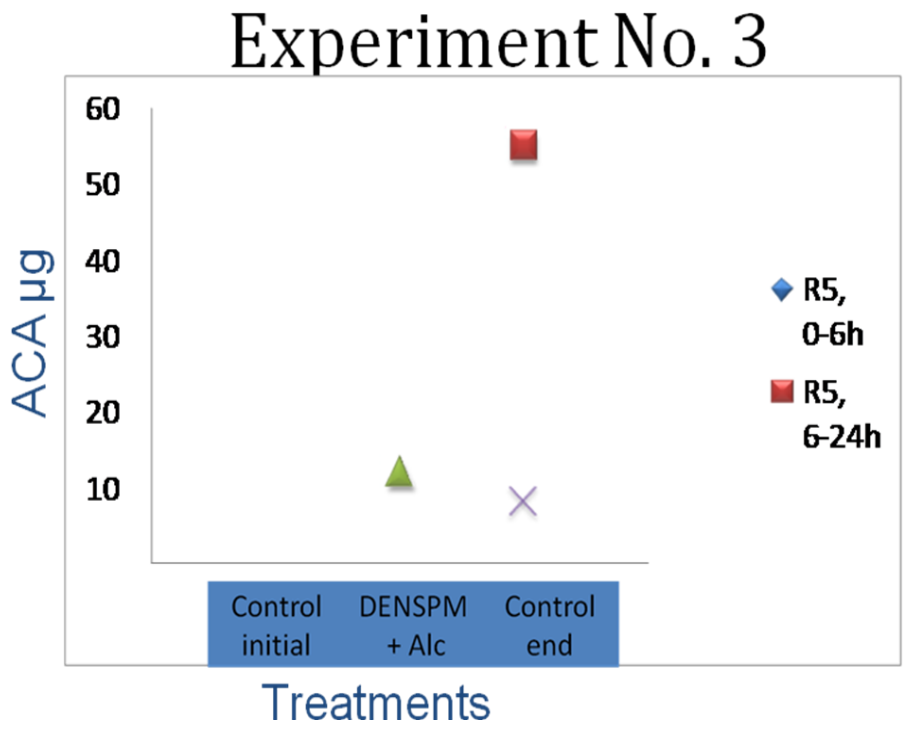


Table 2.3. Numerical data for Figure 3 presented above. Data as total metabolite (ACA) excretion in µg.

Rat (time interval)	Control initial	DENSPM + Alc	Control end
R5, 0-6h			
R5, 6-24h			55
R6, 0-6h		12	
R6, 6-24h			8.17

R5: Rat with ear punch in experiment 3
R6: Rat without ear punch in experiment 3

Figure 2.4. Acetylamantadine (ACA) excreted (µg , 0-24 h urine) vs. treatment after amantadine HCl (3.0 mg/kg).

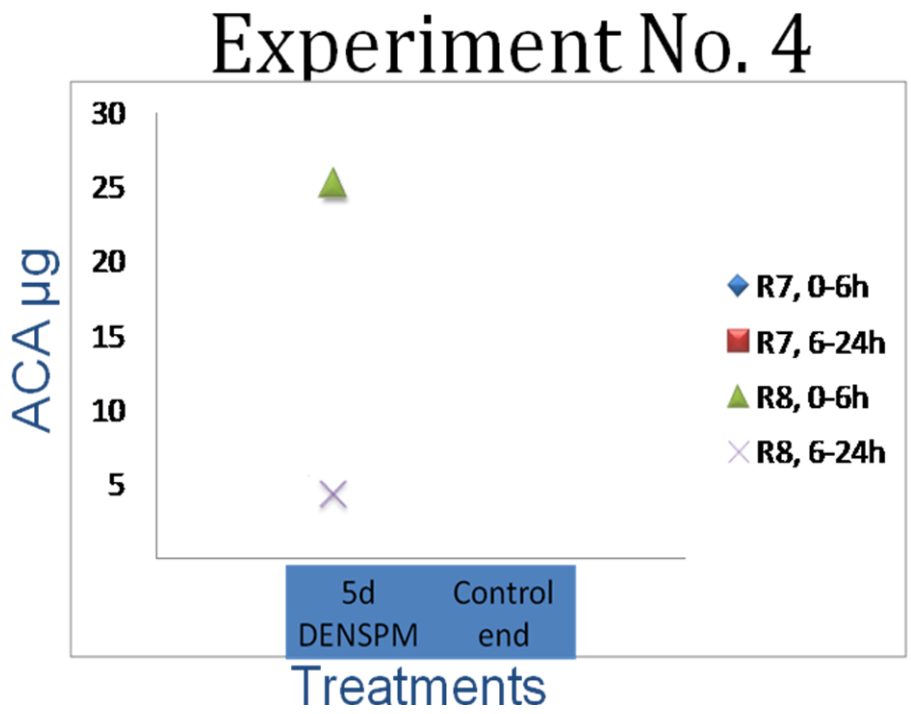


Table 2.4. Numerical data for Figure 4 presented above. Data as total metabolite (ACA) excretion in µg.

Rat (time interval)	5 days DENSPM	Control End
R7, 0-6h		
R7, 6-24h		
R8, 0-6h	25.2	
R8, 6-24h	4.29	

R7: Rat with ear punch in experiment 4
R8: Rat without ear punch in experiment 4

The third and fifth experiments are similar, except in the duration of exposure to Alc. In experiment 3 (Fig. 2.3 and Table 2.3), short exposure to different concentrations of Alc (11 days in total), two days at each concentration of 5%, 10%, and 15% Alc (v/v) respectively and then DENSPM + 15% Alc for another 5 days increased the amount of ACA much less than occurred in the 5th experiment (Fig. 5 and Table 5) where we exposed the rats for one week at each of 5%, 10%, and 15% Alc (v/v) respectively, and then DENSPM + 15% Alc for another 5 days. In both experiments the post intervention control showed no detectable, or trace, amount of ACA in the urine, except in one result in experiment 3 in the 6-24 h urine sample where there was an unexpectedly high amount of ACA during the control post intervention (55 µg) (n=4).

Induction by DENSPM alone produced more ACA in rat urine than the amount produced by exposure to Alc alone. In experiments 1 and 5, increase in Alc concentration led to an increase in ACA production, 5% Alc (5.3 ± 5.7 µg ACA; n=5), 10% Alc (6.1 ± 5.9 µg ACA; n=3), 15% Alc (7.0 ± 6.7 µg ACA; n=3). The very high amount of ACA in the fifth experiment (204 µg) indicates that combined exposure of rats to DENSPM and Alc results in a greater increase in SSAT1 activity than produced by each agent alone.

Figure 2.5. Acetylamantadine (ACA) excreted (µg, 0-24 h urine) vs. treatment after amantadine HCl (3.0 mg/kg).

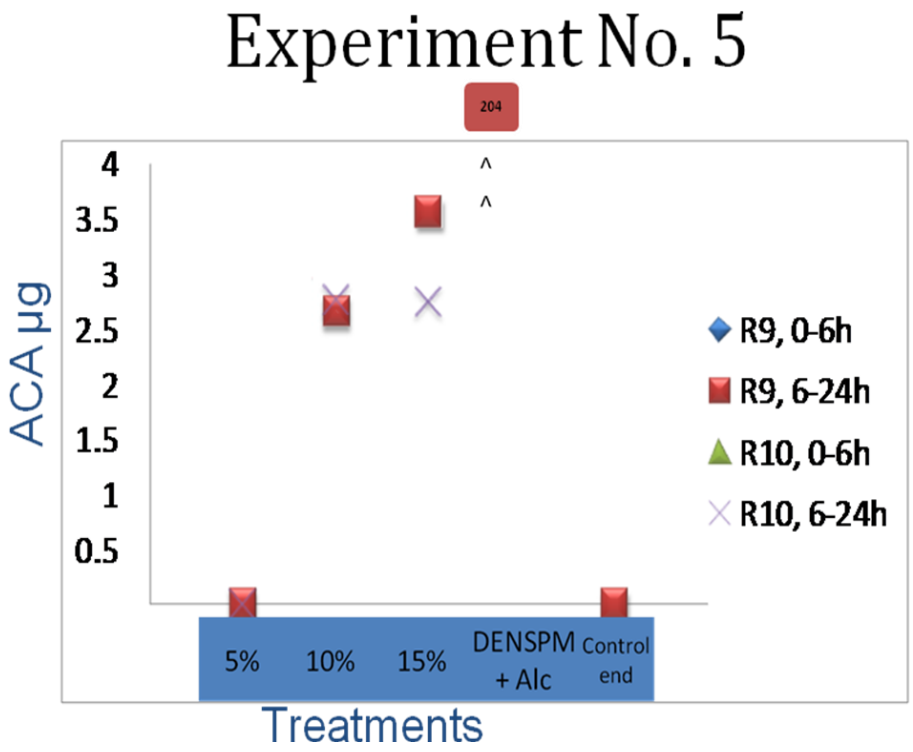


Table 2.5. Numerical data for Figure 5 presented above. Data as total metabolite (ACA) excretion in µg.

Rat (time interval)	5% Alc	10% Alc	15% Alc	DENSPM +15% Alc	Control end
R9, 0-6h					
R9, 6-24h	0	2.66	3.56	204	0
R10, 0-6h					
R10, 6-24h	0	2.76	2.75		

R9: Rat with ear punch in experiment 5
R10: Rat without ear punch in experiment 5

In experiments 2 and 4 when DENSPM was administered without Alc for 5 days, there was a reduction in rat weight in the first day after the first DENSPM injection, which was also seen in some rats in the 1st day after the 1st amantadine injection. The rats then started gaining weight gradually, beginning on the second day after the first DENSPM injection. The rate of weight gain was slower than the rate observed during the control or Alc administration period (Tables A2.3, A2.4, A2.7, & A2.8).

The combined administration of DENSPM and 15% v/v Alc caused the maximum reduction in rat weight. There was no weight gain in the 5 days of combined administration of DENSPM and Alc and the weight loss varied between 2-15 g/D (Tables A2.5, A2.6, A2.9, & A2.10).

2.4. DISCUSSION

This pilot study has demonstrated that Alc is able to induce SSAT1 activity *in vivo* in male rats, as detected by acetylation of amantadine HCl excreted in the urine. This effect appears to be dose-dependent, as reflected by ACA excretion in the urine. DENSPM is a more powerful inducer of SSAT1, as quantified by ACA elimination in urine. The combination of Alc and DENSPM produce more ACA elimination than either agent alone suggesting that there is a possibility of synergy between Alc and DENSPM.

Initially studies indicated that amantadine was not metabolized in humans (Bleidner, Harmon et al. 1965). ACA was first detected in the urine of three healthy young male volunteers who ingested a 200 mg dose of amantadine. It was reported that 5-15% of the administered dose was recovered in the urine in acetylated form (Koppel and Tenczer 1985). As a drug with a primary amino group, it was anticipated to be metabolized by NAT1 or NAT2, but it was subsequently proven that amantadine is not acetylated by either of these two acetyltransferase enzymes (Bras et al., 1998). Later it was demonstrated that amantadine was acetylated by SSAT1, a previously unrecognized drug acetylating enzyme. Normal animal liver tissue does not produce sufficient SSAT1 for detection of its contribution to drug metabolism studies using our previous, less sensitive, analytical methods. SSAT1 is present in very small amounts in mammalian cells and is highly inducible. In rat hepatocytes, it has been estimated that less than 1000 molecules are present compared to 60,000 molecules in an induced cell (Pegg, Seely et al. 1982). It was demonstrated that for SSAT1 acetylation of amantadine to be detected, it must be induced from its basal level. Since amantadine was specific for SSAT1, it

could be used to distinguish acetylation that is proceeding by NAT1 or NAT2 from SSAT1 (Bras, Janne et al. 2001).

Our first hypothesis declared that chronic exposure to Alc in drinking water will increase SSAT1 in the rat liver and will be reflected by the presence of ACA in rat urine. SSAT1 is a stress enzyme and increasing the concentration of Alc in the drinking water could be exposing the rats to more stress which eventually will induce SSAT1. From the results, our data supported this hypothesis.

The results from the samples that were analyzed by LC/MS/MS analysis were more sensitive and accurate. Although initial GLC analysis results were promising, proceeding with the more sensitive LC/MS/MS analysis method required repeated analysis. The loss of some study samples from initial GLC analysis resulted in the inability to reanalyze all rat urine samples with the more sensitive technique. Our new more sensitive LC/MS/MS assay is now capable of detecting quantities of ACA that were not possible by the previously utilized GLC assay (Bras, Hoff et al. 1998). In the first experiment, the observation of the presence of either small or non-detectable amount of ACA in the pre intervention control is consistent with previous data indicating that SSAT1 is present in a very small amount in mammalian cells, and normal animal livers do not produce sufficient SSAT1 for drug metabolism studies (Pegg, Seely et al. 1982).

The finding that the rate of ACA produced/h is more in 0 - 6 h samples might be explained by the short plasma half-life of amantadine in rats (mean $t_{1/2}$ = 8.6 h) (Goralski, Smyth et al. 1999). In experiments 1 and 5, the increase in ACA production with the increase of Alc concentration administered to the rats have qualitatively similar

result to those in a previous paper describing induction of SSAT1 in rats by chronic administration of alcohol (Perin and Sessa 1993). In their experiments, rats were fed a nutritionally complete liquid diet with 36% or 12 % of total calories supplied as Alc or isocaloric carbohydrates for 4 months. Chronic administration of high dose ethanol (36% of calories) increased polyamine acetylation in liver by stimulation of SSAT1 activity. Such stimulation didn't occur in rats on the lower dose Alc diet (12% of calories). Putrescine and spermidine increased by about 70% (from 7 ± 1 to 12 ± 2 μg) and 40% (from 440 ± 31 to 623 ± 63 μg) respectively, whereas spermine was decreased from 909 ± 66 to 623 ± 63 μg. Alc didn't modify the total polyamine content. In our study, the chronic but shorter administration of Alc also increased the activity of SSAT1, which was detected by an increase in the level of ACA in the rat urine.

In the 2nd hypothesis we proposed that DENSPM will induce SSAT1, which will be reflected by the presence of ACA in rat urine. The 4th experiment, in which the protocol is similar to the 2nd experiment supported our 2nd hypothesis and showed an increase in ACA production after DENSPM administration (50 mg/kg) for 5 days. In contrast to our observations in experiment 1, the greater rate of ACA production in 0 - 6 h than in the 6 - 24 h might be due to the fact that DENSPM is a more powerful SSAT1 inducer than Alc, as reflected by the higher amount of ACA production than Alc after induction. Polyamine analogues such as DENSPM induce the activity of SSAT1 by number of complex regulatory mechanisms, which involve stabilization of the enzyme protein and accumulation of SSAT1 mRNA (Fogel-Petrovic, Kramer et al. 1997), allowing further acetylation of amantadine.

The third hypothesis that was supported by experiments 3 and 5 assumed that combined exposure of rats to DENSPM and Alc will result in a greater increase of liver SSAT1 than produced by the individual substances. The finding that there is more production of ACA with combined administration of Alc and DENSPM with longer exposure to Alc before DENSPM administration (Experiment 5) than shorter exposure to Alc (experiment 3) and exposure to DENSPM alone (experiment 4), is consistent with the fact that SSAT1 is a stress enzyme and increasing the concentration and duration of exposure to Alc in the drinking water could be exposing the rats to more stress. The data suggest a possible synergistic interaction between Alc and DENSPM, which was reflected by increasing ACA excreted in the urine in experiment 5 (204 µg). However, in this study we cannot rule out the effect of weight loss on the induction of SSAT1. The greatest weight loss occurred with longer exposure to Alc combined with 5 days DENSPM, and might be an additional stress factor that induced SSAT1 activity.

In the post intervention control, the return of the ACA levels to either trace or non-detectable amounts in the rat urine is consistent with the assertion that SSAT1 is a stress enzyme, which can be induced and suppressed quickly, as evaluated by the extent of ACA production in rat urine. For one result in experiment 3, there was an unexpectedly high amount of ACA at post intervention control in 6-24 h (55 µg), which may be explained by liver damage and/or weight loss with concurrent Alc and DENSPM exposure in that rat.

Our study is consistent with the other studies that reported induction of some drug acetylation with Alc ingestion in human and rats (Olsen and Morland 1978; Olsen and Morland 1982) (Thomas and Solomonraj 1977). These observations and other similar results suggest that SSAT1 induction by Alc may explain increased acetylation of NAT2- selective substrates, which is a testable hypothesis. Initial enzymatic studies from our own laboratory support this possibility (Bras, Janne et al. 2001).

Amantadine could be used to differentiate between acetylation by SSAT1 and NATs, since it is not subject to acetylation by either NAT1 or NAT2 (Bras, Hoff et al. 1998). As mentioned in the introduction, it has been reported that drug acetylation and SSAT1 levels and activity are increased in persons with a diagnosis of cancer (Russell 1971; Suh, Lee et al. 1997). Amantadine could be used to determine if there are increased levels of SSAT1 activity in the body that could be a marker that predicts malignancy (Sitar and Bras 2004).

In the future, studies are required to determine if other NAT1 and NAT 2 substrates (arylamines, hydrazines, and primary amines) are acetylated by SSAT1 or if SSAT1, when induced, is a contributor to acetylation of amino-containing drugs. Our new model of SSAT1 induction using Alc and DENSPM in male Sprague Dawley rats is less expensive than transgenic mice and can now be optimized using a larger number of animals in further studies. Understanding factors that modify drug metabolism should facilitate optimization of drug therapy, especially when the enzyme of interest is altered in disease states and/or by environmental exposure.

3. GENERAL DISCUSSION

Both studies presented in this thesis have supported the proposed hypotheses. In the human study, bicarbonate administration was able to impair amantadine renal secretion while creatinine and its clearance were not affected, indicating that bicarbonate was modifying the secretory component of amantadine renal elimination. In the rat study, Alc was able to induce SSAT1 activity *in vivo* in male rats, as detected by ACA excretion in the urine. This effect was duration and dose-dependent. DENSPM is a more powerful inducer of SSAT1 in this rat model, as quantified by ACA elimination in urine. The combination of Alc and DENSPM produced more urinary ACA than either agent alone and the expected amount when both agents were administered concurrently, suggesting that there is a likelihood of synergy between Alc and DENSPM.

The results of an acute bicarbonate load administered to healthy male volunteers were consistent with previous observations in rats (Goralski, Smyth et al. 1999), dogs (Sitar, Escobar et al. 1997), and *in vitro* renal cortical distal and proximal tubule studies (Escobar, Wong et al. 1994). Also our results on induction of SSAT1 activity in rats were relative and comparable to those of previous similar studies that investigated the induction of SSAT1 using Alc ingestion on transgenic rats (Perin and Sessa 1993) and the DENSPM effect *in vivo* and *in vitro* (Pegg, Wechter et al. 1989; Kramer, Fogel-Petrovic et al. 1997; Wolff, Armstrong et al. 2003).

Bicarbonate is often administered to intensive care patients to manage acid-base imbalance due to chronic or acute illness. Aging might result in increased plasma bicarbonate levels that might impair renal elimination of amantadine and possibly other organic cation drugs, especially those that are handled by bicarbonate-dependent OCTs

in the kidney. More than 30% of drugs are organic cations, and some of them are highly toxic to the kidney such as gentamicin (Bennett 1989). Alteration in those drugs disposition might change their efficacy and safety. The mechanisms of renal OCTs regulation remain incompletely understood, and further studies will be necessary to define more precisely both transporter regulation and subsequent consequences for cationic drug elimination by the kidney.

Reproducing the rat study with a larger sample size using the new more sensitive LC/MS/MS will define more precisely the relationship between SSAT1 induction and Alc and DENSPM dose. Further refining of this animal model will provide an efficient and economical basis to further investigate other xenobiotics that have been reported to increase their acetylated metabolite excretion with Alc ingestion, such as isoniazid and procainamide (Thomas and Solomonraj 1977; Olsen and Morland 1982). Generally, NAT1 and NAT 2 substrates (arylamines, hydrazines, and primary amines) are reasonable candidates for further investigation.

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5. APPENDIX

Table A1.1. Amantadine:creatinine clearance ratio calculation.

Vol	Treat-ment	A plasma conc.	A urine conc (ng/mL)	Urine volume (ml)	AUC (ng.min/mL)	A Cl_r (mL/min)	Cl_{cr} (mL/min)	ACl_r:Cl_{cr} Ratio
1	Bicarb	256						
		228	21144	310	29771	220	201	1.10
		215	8503	430	26990	135	135	1.00
		220	3177	690	27141	81	148	0.55
		221	7871	490	26909	143	227	0.63
	Saline	284						
		247	23819	480	35089	326	177	1.84
		237	8502	1080	31012	296	120	2.47
		230	24935	200	28032	178	104	1.71
		217	25770	220	26833	211	72	2.93
2	Bicarb	334						
		293	9740	480	39172	119	117	1.02
		260	2718	480	34553	38	151	0.25
		261	4016	340	29972	46	116	0.39
		269	2090	1360	31793	89	178	0.50
	Saline	282						
		273	7543	560	33280	127	62	2.05
		278	5461	1600	34398	254	35	7.26
		258	15085	430	32146	202	131	1.54
		248	17725	250	29116	152	122	1.25

Vol.	Treat-ment	A plasma conc.	A urine conc (ng/mL)	Urine volume (ml)	AUC (ng.min/mL)	A Cl_r (mL/min)	Cl_{cr} (mL/min)	ACl_r:Cl_{cr} Ratio
3	Saline	249						
		223	12408	610	30696	247	96	2.57
		177	9195	900	24018	345	112	3.08
		175	22110	530	20244	579	194	2.98
		146	7000	820	18416	312	244	1.28
	Bicarb	261						
		218	4308	770	27537	120	55	2.19
		188	4135	1010	25379	165	118	1.39
		184	3891	570	21363	104	155	0.67
		175	2897	620	21546	83	159	0.52
4	Bicarb	523						
		517	4857	810	57221	69	64	1.07
		461	1229	1100	58666	23	114	0.20
		447	2805	710	50817	39	60	0.65
		418	3607	780	55356	51	49	1.04
	Saline	443						
		397	32382	400	50411	257	124	2.07
		367	6674	940	45816	137	110	1.24
		319	5280	850	41117	109	71	1.54
		289	10945	640	36428	192	167	1.15

Vol	Treat-ment	A plasma conc.	A urine conc (ng/mL)	Urine volume (ml)	AUC (ng.min/mL)	A Cl_r (mL/min)	Cl_{cr} (mL/min)	ACl_r:Cl_{cr} Ratio
5	Saline	244						
		219	16750	530	30068	295	142	2.079
		196	13229	700	25902	358	151	2.37
		168	15891	550	20904	418	253	1.65
		143	10380	510	18661	284	135	2.10
	Bicarb	208						
		197	20596	290	23272	257	119	2.16
		154	6694	740	21051	235	129	1.82
		155	6460	320	18569	111	179	0.62
		162	7476	350	19043	137	152	0.90
6	Saline	297						
		297	8545	490	37682	111	94	1.18
		274	12408	690	34231	250	93	2.69
		239	28515	280	29494	271	242	1.12
		214	25872	300	26071	298	75	3.97
	Bicarb	298						
		304	16431	188	36104	86	96	0.89
		248	2064	1680	33075	105	106	0.99
		260	2815	380	29191	37	99	0.37
		255	5843	420	28319	87	120	0.72

Vol	Treat-ment	A plasma conc.	A urine conc (ng/mL)	Urine volume (ml)	AUC (ng.min/mL)	A Cl_r (mL/min)	Cl_{cr} (mL/min)	ACl_r:Cl_{cr} Ratio
7	Bicarb	190						
		170	5901	1350	20702	385	199	1.93
		147	4767	970	19831	233	164	1.42
		180	8262	420	18815	184	122	1.51
		153	5560	600	19139	174	193	0.90
	Saline	196						
		172	22507	300	23036	293	64	4.58
		162	10434	1090	21706	524	157	3.34
		155	6708	220	15814	93	42	2.22
		125	5749	1060	16767	363	178	2.04
8	Bicarb	386						
		371	72954	120	45403	193	229	0.84
		324	5833	830	52061	93	107	0.87
		318	7907	180	33691	42	214	0.20
		323	7382	540	33635	119	91	1.30
	Saline	345						
		320	10946	550	39903	151	53	2.85
		296	8642	600	36944	140	117	1.20
		265	62415	100	26604	235	138	1.70
		219	20568	350	30248	238	45	5.29

Vol.	Treat-ment	A plasma conc.	A urine conc (ng/mL)	Urine volume (ml)	AUC (ng.min/mL)	A Cl_r (mL/min)	Cl_{cr} (mL/min)	ACl_r:Cl_{cr} Ratio
9	Saline	441						
		373	69817	200	50866	275	139	1.97
		359	96960	120	45769	254	143	1.78
		300	52191	170	37922	234	126	1.86
		313	38127	140	33729	158	139	1.14
	Bicarb	382						
		366	165449	80	39244	337	161	2.09
		334	4920	770	43745	87	158	0.55
		321	4553	410	29475	63	100	0.63
		304	8298	250	37499	55	211	0.26
10	Saline	304						
		286	3768	625	32448	73	64	1.13
		234	5088	950	32526	149	93	1.60
		250	13183	400	25405	208	143	1.45
		220	20910	180	30526	123	156	0.79
	Bicarb	279						
		269	25808	170	31533	139	152	0.92
		236	2495	1040	30293	86	107	0.80
		245	1601	990	27652	57	122	0.47
		247	6223	440	31989	86	171	0.50

Vol.	Treat-ment	A plasma conc.	A urine conc (ng/mL)	Urine volume (ml)	AUC (ng.min/mL)	A Cl_r (mL/min)	Cl_{cr} (mL/min)	ACl_r:Cl_{cr} Ratio
11	Bicarb	242						
		225	33646	270	26844	338	119	2.84
		189	3627	1160	24842	169	209	0.81
		185	2331	670	20544	76	169	0.45
		185	3041	470	20337	70	174	0.40
	Saline	257						
		216	77719	70	26038	209	81	2.58
		200	12748	680	24972	347	181	1.92
		163	16552	590	27182	359	131	2.74
		152	17753	350	14156	439	218	2.01
12	Saline	334						
		341	24446	260	37128	171	98	1.75
		285	9004	625	36006	156	149	1.05
		269	5310	590	30478	103	138	0.74
		261	10682	460	29179	168	137	1.23
	Bicarb	298						
		264	56013	80	29499	152	135	1.13
		248	5118	870	32045	139	150	0.93
		264	2196	870	37122	51	116	0.44
		253	5687	250	23225	61	147	0.42

Vol. : Volunteer
A : Amantadine
Conc : Concentration
AUC : Area under the curve
Cl_r : Renal clearance
Cl_{cr} : Creatinine clearance

Table A1.2. Area under the curve calculation.

Volunteers	Treatment	Time (min)	Plasma Conc (ng/mL)	AUC (ng.min/mL)
1	Bicarb		256	
		123	228	29766
		122	214	26962
		125	220	27125
		122	221	26901
	Saline		284	
		132	247	35046
		128	237	30976
		120	230	28020
		120	217	26820
2	Bicarb		334	
		125	293	39188
		125	260	34563
		115	261	29958
		120	268	31740
	Saline		282	
		120	272	33240
		125	277	34313
		120	257	32040
		115	248	29038
3	Saline		249	
		130	222	30615
		120	177	23940
		115	174	20183
		115	145	18343
	Bicarb		260	
		115	218	27485
		125	187	25313
		115	183	21275
		120	175	21480
4	Bicarb		523	
		110	517	57200
		120	461	58680
		112	447	50848
		128	418	55360
	Saline		443	
		120	397	50400
		120	367	45840
		120	318	41100
		120	289	36420
5	Saline		244	

Volunteers	Treatment	Time (min)	Plasma Conc (ng/mL)	AUC (ng.min/mL)
		130	219	30095
		125	196	25938
		115	168	20930
		120	143	18660
	Bicarb		208	
		115	197	23288
		120	154	21060
		120	155	18540
		120	162	19020
6	Saline		296	
		127	297	37656
		120	274	34260
		115	239	29498
		115	214	26048
	Bicarb		298	
		120	303	36060
		120	248	33060
		115	260	29210
		110	255	28325
7	Bicarb		190	
		115	170	20700
		125	147	19813
		115	180	18803
		115	153	19148
	Saline		196	
		125	172	23000
		130	162	21710
		100	154	15800
		120	125	16740
8	Bicarb		386	
		120	370	45360
		150	324	52050
		105	318	33705
		105	322	33600
	Saline		345	
		120	320	39900
		120	295	36900
		95	264	26553
		125	219	30188

Volunteers	Treatment	Time (min)	Plasma Conc (ng/mL)	AUC (ng.min/mL)
9	Saline		441	
		125	373	50875
		125	360	45813
		115	300	37950
		110	313	33715
	Bicarb		382	
		105	366	39270
		125	334	43750
		90	321	29475
		120	304	37500
10	Saline		304	
		110	286	32450
		125	234	32500
		105	250	25410
		130	220	30550
	Bicarb		279	
		115	269	31510
		120	236	30300
		115	245	27658
		130	247	31980
11	Bicarb		242	
		115	225	26853
		120	189	24840
		110	184	20515
		110	185	20295
	Saline		257	
		110	216	26015
		120	200	24960
		150	163	27225
		90	152	14175
12	Saline		334	
		110	341	37125
		115	285	35995
		110	269	30470
		110	261	29150
	Bicarb		297	
		105	264	29453
		125	248	32000
		145	263	37048
		90	252	23175

Conc: concentration
AUC: area under the curve

Table A2.1. Summary of experimental procedures for rat number 1.

Date (DD/MM)	Time (h)	Weight (g)	A-HCl Dose (mL)	Urine volume 0 – 6 h (mL)	Urine volume 6 – 24 h (mL)	Urine pH 6 – 24 h	Fluid intake
25/04	09:40	246	0.25	3.45			Distilled water
26/04	09:40	246			21.5	7.60	5% v/v ethanol
27/04-01/05	10:30	252-275					5% v/v ethanol
02/05	10:30	283	0.28	2.30			5% v/v ethanol
03/05	10:40	284			15.5	7.44	10% v/v ethanol
04/05-08-05	10:40	286-310					10% v/v ethanol
09/05	09:40	317	0.32	2.25			10% v/v ethanol
10/05	09:40	324			15.2	7.07	15% v/v ethanol
11/05-14/05	10:00	320-336					15% v/v ethanol
16/05	10:00	346	0.35	1.50			15% v/v ethanol
17/05	11:00	350			14.0	7.33	Distilled water
18/05-19/05	11:00	348-353					Distilled water
23/05	10:15	373	0.37	4.30			Distilled water
24/05	10:15	378			27.0	8.00	Distilled water

Table A2.2. Summary of experimental procedures for rat number 2.

Date (DD/MM)	Time (h)	Weight (g)	A- HCl Dose (mL)	Urine volume 0-6 h (mL)	Urine volume 6-24 h (mL)	Urine pH 6-24 h	Fluid intake
25/04	9:40	252	0.25	2.8			Distilled water
26/04	9:40	249			26.5	7.65	5% v/v ethanol
27/04-01/05	10:30	257-302					5% v/v ethanol
01/05	10:30	310	0.31	3			5% v/v ethanol
03/05	10:40	311			18.5	7.47	10% v/v ethanol
04/05-08/05	10:40	317-347					10% v/v ethanol
09/05	9:40	358	0.36	3.4			10% v/v ethanol
10/05	9:40	357			16.5	6.73	15% v/v ethanol
11/05-14/05	11:00	362-386					15% v/v ethanol
16/05	10:00	386	0.39	1.85			15% v/v ethanol
17/05	11:00	392			14	6.87	Distilled water
18/05-19/05	11:00	404-410					Distilled water
23/05	10:15	438	0.44	5.7			Distilled water
24/05	10:15	445			29.2	7.23	Distilled water

Table A2.3. Summary of experimental procedures for rat number 3.

Date (DD/MM)	Time (h)	weight (g)	DENSPM Dose (mL)	A-HCl Dose (mL)	Urine volume 0-6 h (mL)	Urine volume 6-24 h (mL)	Urine pH 6-24 h	Fluid Intake
12/06	9:30	310	0.36					Distilled water
13/06	9:30	288	0.34					Distilled water
14/06	10:00	310	0.36					Distilled water
15/06	9:30	310	0.36					Distilled water
16/06	10:00	320	0.37		8.3			Distilled water
	11:00			0.32				
17/06	9:30	331				41	8.12	Distilled water
20/06-26/06	Cont-rol	340-388						Distilled water
27/06	10:00	394		0.39	1			Distilled water
28/06	10:00	398				34.3	6.72	Distilled water

Table A2.4. Summary of experimental procedures for rat number 4.

Date (DD/MM)	Time (h)	Weight (g)	DENSPM Dose (mL)	A-HCl Dose (mL)	Urine volume 0-6 h (mL)	Urine volume 6-24 h (mL)	Urine PH 6-24 h	Fluid Intake
12/06	9:30	285	0.33					Distilled water
13/06	9:30	265	0.3					Distilled water
14/06	9:30	288	0.34					Distilled water
15/06	9:30	290	0.34					Distilled water
16/06	9:30	300	0.35		8			Distilled water
	10:30			0.3				
17/06	Cont-rol	309				45.5	8.09	Distilled water
20/06-26/06	10:00	320-364						Distilled water
27/06	10:00	378		0.38	1.4			Distilled water
28/06	10:00	385				23	7.26	Distilled water

Table A2.5. Summary of experimental procedures for rat number 5.

Date (DD/MM)	Time (h)	weight (g)	DENSPM Dose (mL)	A-HCl Dose (mL)	Urine volume 0-6 h (mL)	Urine volume 6-24 h (mL)	Urine pH 6-24 h	Fluid Intake
27/06-28/06	10:00	241-250						5% v/v ethanol
29/06-30/06	10:00	258-250						10% v/v ethanol
01/07-02/07	10:00	260-266						15% v/v ethanol
03/07	9:00	275	0.32					15% v/v ethanol
04/07	9:00	265	0.31					15% v/v ethanol
05/07	9:30	256	0.30					15% v/v ethanol
06/07	9:00	245	0.28					15% v/v ethanol
07/07	9:00	237	0.27		0.75			15% v/v ethanol
	10:00			0.24				
08/07	10:00	230				9.5	8	Distilled water
09/07-13/07	10:00	251-319						Distilled water
14/07	10:00	328		0.33	3.1			Distilled water
15/07	10:00	333				27.5	7.3	Distilled water

Table A2.6. Summary of experimental procedures for rat number 6.

Date (DD/MM)	Time (h)	Weight (g)	DENSPM Dose (mL)	A-HCl Dose (mL)	Urine volume 0-6 h (mL)	Urine volume 6-24 h (mL)	Urine pH 6-24 h	Fluid Intake
27/06-28/06	10:00	243-252						5% v/v ethanol
29/06-30/06	10:00	263-252						10% v/v ethanol
01/07-02/07	10:00	265-273						15% v/v ethanol
03/07	9:00	280	0.32					15% v/v ethanol
04/07	9:00	265	0.31					15% v/v ethanol
05/07	9:00	254	0.29					15% v/v ethanol
06/07	9:00	257	0.3					15% v/v ethanol
07/07	9:00	257	0.3		1			15% v/v ethanol
	10:00			0.26				
08/07-	10:00	264				9.5	7.8	Distilled water
09/07-13/07	10:00	280-335						Distilled water
14/07	10:00	340		0.34	2.8			Distilled water
15/07	10:00	345				43	7.5	Distilled water

Table A2.7. Summary of experimental procedures for rat number 7.

Date (DD/MM)	Time (h)	Weight (g)	DENSPM Dose (mL)	A-HCl Dose (mL)	Urine volume 0-6 h (mL)	Urine volume 6-24 h (mL)	Urine pH 6-24 (h)	Fluid Intake
31/07	10:10	225	0.26					Distilled water
01/08	9:35	226	0.26					Distilled water
02/08	10:00	232	0.27					Distilled water
03/08	9:30	233	0.27					Distilled water
04/08	10:00	241	0.28		9ml			Distilled water
	11:00			0.24				
05/08	9:30	250				14ml	8	Distilled water

Table A2.8. Summary of experimental procedures for rat number 8.

Date (DD/MM)	Time (h)	Weight (g)	DENSPM Dose (mL)	A-HCl Dose (mL)	Urine volume 0-6 h (mL)	Urine volume 6-24 h (mL)	Urine pH 6-24 (h)	Fluid Intake
31/07	10:10	232	0.27					Distilled water
01/08	9:35	235	0.27					Distilled water
02/08	10:00	240	0.28					Distilled water
03/08	9:30	244	0.28					Distilled water
04/08	10:00	250	0.29		14			Distilled water
	11:00			0.24				
05/08	9:30	258				39	7.8	Distilled water

Table A2.9. Summary of experimental procedures for rat number 9.

Date (DD/MM)	Time (h)	Weight (g)	DENSPM Dose (mL)	A-HCl Dose (mL)	Urine volume 0-6 h (mL)	Urine volume 6-24 h (mL)	Urine pH 6-24 (h)	Fluid Intake
14/08	09:30	245		0.25	6			5% v/v ethanol
15/08	09:00	250				25	7.0	5% v/v ethanol
16/08-20/08	09:00	255-300						5% v/v ethanol
21/08	09:00	307		0.31	4.5			5% v/v ethanol
22/08	09:00	310				20.25	7.3	10 % v/v ethanol
23/08-28/08	09:00	313-355						10% v/v ethanol
29/08	09:00	362		0.36	2.15			10% v/v ethanol
30/08	09:00	361				14	7.0	15 % v/v ethanol
31/08-05/09	09:00	360-390						15% v/v ethanol
06/09	09:00	398		0.4	1.05			15% v/v ethanol
07/09	09:00	399				15.5	7.04	15% v/v ethanol
08/09-10/09	09:00	411-416						15% v/v ethanol
11/09	09:00	420	0.49					15% v/v ethanol
12/09	09:00	405	0.47					15% v/v ethanol

Cont. Table A2.9. Summary of experimental procedures for rat number 9.

Date (DD/MM)	Time (h)	Weight (g)	DENSPM Dose (mL)	A-HCl Dose (mL)	Urine volume 0-6 h (mL)	Urine volume 6-24 h (mL)	Urine pH 6-24 (h)	Fluid Intake
13/09	09:00	403	0.47					15% v/v ethanol
14/09	09:00	398	0.46					15% v/v ethanol
15/09	09:00	394	0.46	0.4	1.3			15% v/v ethanol
16/09-24/09	09:00	399-460				12	7.26	Distilled water
25/09	09:00	462		0.46	9.5			Distilled water
26/09	09:00	438				47	7.5	Distilled water

Table A2.10. Summary of experimental procedures for rat number 10.

Date (DD/MM)	Time (h)	Weight (g)	DENSPM Dose (mL)	A-HCl Dose (mL)	Urine volume 0-6 h (mL)	Urine volume 6-24 h (mL)	Urine pH 6-24 (h)	Fluid Intake
14/08	09:30	240		0.24	5ml			5% v/v ethanol
15/08	09:00	244				20	7.1	5% v/v ethanol
16/08-20/08	09:00	253-293						5% v/v ethanol
21/08	09:00	303		0.3	4.5			5% v/v ethanol
22/08-	09:00	305				21	7.1	10 % v/v ethanol
23/08-27/08	09:00	311-337						10 % v/v ethanol
29/08	09:00	350		0.35	1.3			10% v/v ethanol
30/08	09:00	359				12	6.64	15 % v/v ethanol
31/08-05/09	09:00	360-386						15 % v/v ethanol
06/09	09:00	397		0.4	2.1			15% v/v ethanol
07/09-	09:00	405				14.5	6.75	15% v/v ethanol
08/09-10/09	09:00	413-416						15% v/v ethanol

Cont. Table A2.10. Summary of experimental procedures for rat number 10.

Date (DD/MM)	Time (h)	Weight (g)	DENSPM Dose (mL)	A-HCl Dose (mL)	Urine volume 0-6 h (mL)	Urine volume 6-24 h (mL)	Urine pH 6-24 (h)	Fluid Intake
11/09	09:00	418	0.48					15% v/v ethanol
12/09	09:00	404	0.47					15% v/v ethanol
13/09	09:00	401	0.46					15% v/v ethanol
14/09	09:00	387	0.45					15% v/v ethanol
15/09	09:00	379	0.44	0.38	1.1			15% v/v ethanol
16/09	09:00	385				10.1	6.93	Distilled water
17/09-24/09	09:00	393-453						Distilled water
25/09	09:00	460		0.46	6.3			Distilled water
26/09	09:00	425				41		Distilled water