Repeated Administration of *d*-Amphetamine Results in a Time-dependent and Dose-independent Sustained Increase in Urinary Excretion of *p*-Hydroxyamphetamine in Mice

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The biotransformation of *d*-amphetamine into *p*-hydroxyamphetamine (HA) by cytochrome P450 occurs in several species besides humans. The extent of HA excretion varies among species and the oxidative pathway involved in this biotransformation is reported to be implicated in the toxic effects of *d*-amphetamine. The aim of this study was to evaluate the influence of dose and repeated administration of *d*-amphetamine on the urinary excretion of *d*-amphetamine and HA in mice. Charles River Caesarian Derived (CD)-1 mice, kept in metabolic cages, were treated with *d*-amphetamine (5, 10 and 20 mg/kg, i.p., daily, for 14 days). Urine was collected at 24 hr intervals and analyzed by HPLC for the quantification of *d*-amphetamine and HA. Urinary excretion of *d*-amphetamine increased in a dose dependent manner, the urinary levels being fairly constant after the 4th day. On the other hand the urinary excretion of HA increased during the whole time of *d*-amphetamine dosing and was not dose dependent. Cortical tubule degeneration was observed for the two higher doses, which may explain the HA excretion pattern, although inhibition of cytochrome P450 (CYP) after high *d*-amphetamine dosing may also be involved.

Key words —— *d*-amphetamine, *p*-hydroxyamphetamine, metabolism, mice, humans

INTRODUCTION

The knowledge about the interspecies differences concerning the pharmacokinetics of drugs of abuse is important to understand the susceptibility to their addictive and toxic effects. Amphetamine is a good example of a drug of abuse with a variable interspecies metabolism. The metabolic profile of this drug, regarding acute administration, was already established in humans and in several laboratory species, revealing some peculiarities. Besides the fact that its metabolic profile is dependent on the species both in a qualitative and in a quantitative way,[1)] it is also known that amphetamine may inhibit cytochrome P450 (CYP) through a nitroso metabolic intermediate, which complexes with the enzyme,[2)] with possible repercussions on its own metabolism. The biotransformation of amphetamine into *p*-hydroxyamphetamine (HA) involves the CYP2D6 isoenzyme as demonstrated both *in vitro*[3)] and *in vivo*.[4)] This oxidative pathway has been shown to be implicated in the toxic effects of amphetamine to isolated rat hepatocytes.[5)]

In spite of the interspecies differences concerning the preferred route of biotransformation, benzylmethylketone, benzoic acid and HA were identified as the main metabolites.[1)] Comparing the human metabolic profile of amphetamine with that of the common experimental animal models, mouse seems to be the species that most resemble humans, the proportion of urinary metabolites being quite analogous. In fact, benzoic acid is the predominant urinary metabolite of amphetamine, both in mice and humans, HA being the second most representative in both species. Thus, during the evaluation of biomarkers of amphetamine exposure in humans or the amphetamine toxicokinetics in mice, besides

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the parent compound, benzoic acid and HA could be the chosen metabolites to quantify. However, since benzoic acid is also an endogenous compound, it should not be used as a biomarker of exposure to amphetamine. On the other hand, the urinary excretion of HA may be not only a biomarker of exposure but can also give an insight of *d*-amphetamine reactivity.[5] Of note, the influence of the dose and/or the repeated administration of *d*-amphetamine on its own metabolism and the urinary elimination of the metabolites are yet to be clarified. Thus, the aim of this study was to evaluate the influence of dose and repeated administration of *d*-amphetamine on the urinary excretion of *d*-amphetamine and HA in the mouse. Liver and kidney histopathology was also evaluated, since amphetamine has been shown to provoke hepatotoxicity[6] and nephrotoxicity,[7,8] and these effects may influence its own metabolism and excretion.

MATERIALS AND METHODS

Materials —— Amphetamine sulfate and HA bromide were kindly supplied by the United Nations Drug Control Program (Vienna, Austria). β-Glucuronidase/arylsulfatase (type H-2, activity 130000 Fishman units/ml) and dabsyl chloride were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Ethyl chloroformate 97% was obtained from Aldrich. All the reagents used were of analytical grade or from the highest available grade.

Animals —— The experiments were carried out under the guidelines of "Principles of laboratory animal care" (NIH publication n° 85–23, revised 1985). Twelve adult male Caesarian Derived (CD)-1 mice (Charles-River, Barcelona, Spain) weighing about 42 g were used in the experiments. The animals were housed individually in metabolic cages, at room temperature of 20 ± 2°C, relative humidity of 50% and 12 hr light/dark cycle (8.00 a.m. on; 8.00 p.m. off). Standard food and water were available *ad libitum*. Water intake, food intake and body weight were monitored daily. *d*-Amphetamine sulfate was dissolved in 0.9% NaCl (saline) and injected i.p. at the doses of 5, 10 and 20 mg/kg (corresponding to 3.67, 7.34 and 14.68 mg/kg free base), daily for 14 days (4 animals per dose). Urine was collected over ice at 24 hr intervals and analysed by HPLC for the quantification of *d*-amphetamine and HA. At the 14th day, the animals were euthanized by cervical dislocation after ethyl ether anesthesia and liver and kidney were processed for histological analysis. Three satellite groups, each with two animals, were treated with the same *d*-amphetamine doses, in order to replace mice that perished during the course of the experiments. In fact, during the course of the experiments, 5 animals perished (one from the dose of 5 mg/kg, day, at the 7th day, two from the dose of 10 mg/kg, day, at the 4th and the 6th day, and one from the higher dose, at the 4th day). There is a possibility that the intraperitoneal administration of *d*-amphetamine may have caused peritonitis in some animals,[9] since 5 animals perished during the course of experiments independently of the dosage. Nevertheless, it is not probable that this possibility had any influence on *d*-amphetamine excretion and/or metabolism, since these pharmacokinetic parameters were similar among animals within groups during the whole experiment.

Urine Analysis —— Extraction of *d*-amphetamine and HA from urine was carried out as previously described.[10] Briefly, 100 µl of urine was diluted with 100 µl of 0.05 M acetate buffer, pH 5.5, and incubated with 20 µl of β-glucuronidase/arylsulfatase for 17 hr at 37°C. Dabsyl chloride reagent (12.4 mM in acetone) was prepared by dissolving 40 mg of dabsyl chloride in 10 ml of acetone by ultrasonic treatment (10 min) and filtering into brown-glass vials, and stored at −20°C. The hydrolysed urine was mixed with 1 ml of 0.15 M $NaHCO_3$ buffer, pH 8.6, stirred and 1 ml of dabsyl chloride added. The drug derivatives were extracted twice with 2 ml of *n*-hexane and some drops of *n*-butanol. The organic layers were combined, evaporated at 40°C, the residue dissolved in methanol and analyzed by HPLC.

HPLC Analysis —— Analysis of the extracts were performed by reverse phase HPLC (HPLC-RP) in a system consisting of a Hewlett Packard (HP, Palo Alto, CA, U.S.A.), HP 1100 Series Quaternary Pump, equipped with a manual injector and a variable wavelength detector. Data and chromatograms were processed by an HP Chemstation for HPLC.

The analytical conditions were as previously described.[10]

Histological Analysis —— Immediately after the sacrifice of the animals, 24 hr after the last dose, liver and kidney fragments were excised and fixed in Bouin's solution, dehydrated in ethanol series and embedded in paraffin for routine histology. Sections 5–7 µm thick were then stained with haematoxylin and eosin for light microscopy observations.

Data Analysis —— All values are expressed as mean ± S.E. Statistical analysis of the data was carried out by Two-Way analysis of variance (ANOVA) and followed by Bonferroni post-hoc test. Statistical significance was accepted at p-values less than 0.05.

RESULTS

The urinary excretion of *d*-amphetamine after administration of *d*-amphetamine sulfate (5, 10 and 20 mg/kg, day i.p. for 14 days) was clearly dose dependent and reached a fairly steady-state after the third day of administration (Fig. 1). Following the third day, the average urinary excretion of *d*-amphetamine differed significantly ($p < 0.01$) among the three groups: 146 ± 13, 349 ± 24, and 601 ± 23 nmol/100 g body weight, daily, respectively.

The urinary excretion of HA after administration of *d*-amphetamine sulfate is represented in Fig. 2. The excretion from day 1 to day 9 showed no statistical differences among the three tested groups although a tendency for a lower excretion of HA was observed for the higher dose group. In the last five days a clear and significant higher excretion of HA was observed for the group dosed with 10 mg/kg, day. Noteworthy, the urinary excretion of HA increased progressively during the 14 days of testing, the ratio between the 14th day and the 3rd day: about 4×, 12× and 7× for the groups dosed with 5, 10 and 20 mg/kg, day, respectively.

The well-known anorectic effect of *d*-amphetamine is shown in Fig. 3 as a decrease in food intake following *d*-amphetamine administration. However, mice developed tolerance to this effect and food intake approached initial values after the 5th day. No differences were found among the three tested groups.

Water intake decreased in mice treated with the two higher doses; however, this effect was transient. No differences were found among the tested groups, although a tendency for a lower water intake was noticed on the 10 mg/kg, day group in the last 5 days (Fig. 4).

Urinary volume was similar between the two

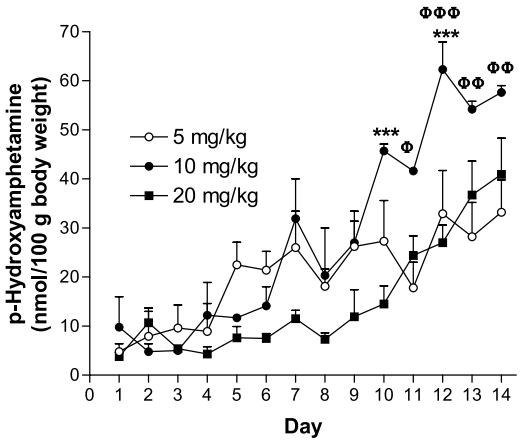

Fig. 2. Urinary Excretion of HA, by *d*-Amphetamine-Treated Mice (i.p., 5, 10 and 20 mg/kg, day, for 14 days)

The values are reported as means ± S.E., $n = 4$; $^{***}p < 0.05$ comparatively to the 5 mg/kg, day group. $\Phi p < 0.05$, $\Phi\Phi p < 0.01$, and $\Phi\Phi\Phi p < 0.001$, comparatively to the 20 mg/kg, day group.

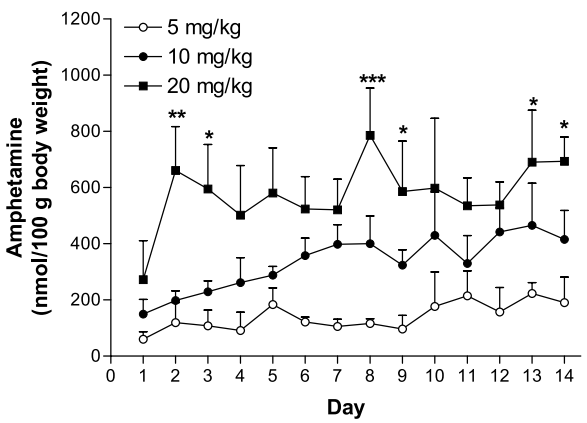

Fig. 1. Urinary Excretion of Amphetamine, by *d*-Amphetamine-Treated Mice (i.p., 5, 10 and 20 mg/kg, day, for 14 days)

The values are reported as means ± S.E., $n = 4$; $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$, comparatively to the 5 mg/kg, day group.

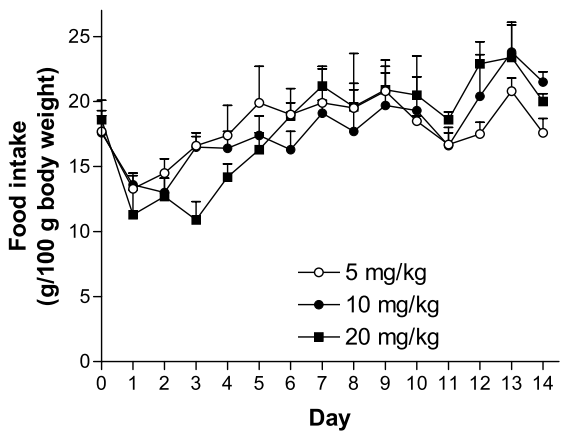

Fig. 3. Food Intake by *d*-Amphetamine-Treated Mice (i.p., 5, 10 and 20 mg/kg, day, for 14 days)

The values are reported as means ± S.E., $n = 4$.

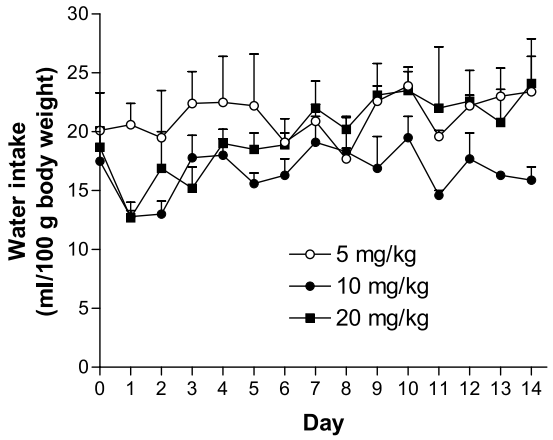

Fig. 4. Water Intake by *d*-Amphetamine-Treated Mice (i.p., 5, 10 and 20 mg/kg, day, for 14 days)
The values are reported as means ± S.E., $n = 4$.

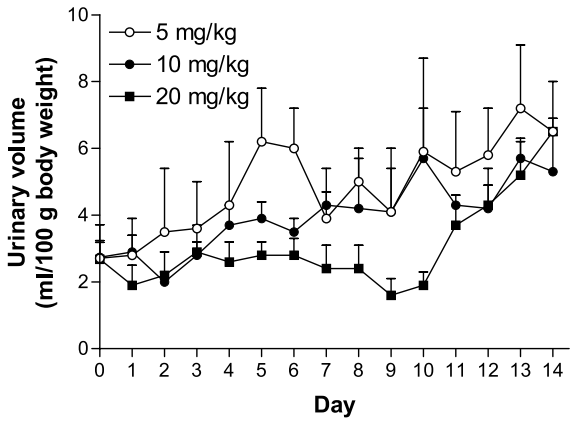

Fig. 5. Urinary Volume Output by *d*-Amphetamine-Treated Mice (i.p., 5, 10 and 20 mg/kg, day, for 14 days)
The values are reported as means ± S.E., $n = 4$.

lower dose groups, with a tendency for a lower urine production being observed in the group dosed with 20 mg/kg, day (Fig. 5).

Body weight showed a tendency for decreasing in a concentration and time dependent manner, especially for the 20 mg/kg dose (Fig. 6).

Liver histology of *d*-amphetamine-treated mice showed normal morphology at all doses (data not shown). Kidney histology of 5 mg/kg *d*-amphetamine-treated mice also showed normal morphology. However, kidney from 10 mg/kg and 20 mg/kg *d*-amphetamine-treated mice revealed cortical tubule degeneration, with higher incidence at 20 mg/kg group, as shown in Fig. 7 and Table 1.

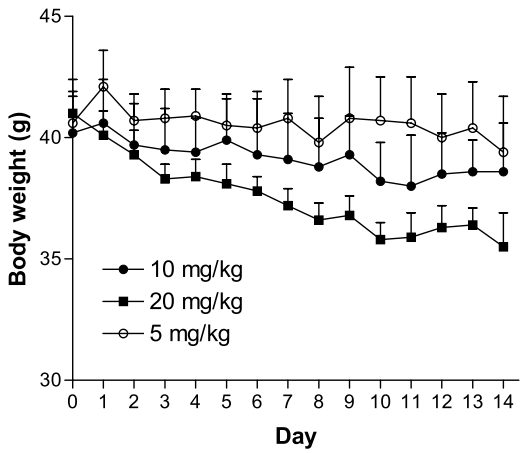

Fig. 6. Body Weight Variation in *d*-Amphetamine-Treated Mice (i.p., 5, 10 and 20 mg/kg, day, for 14 days)
The values are reported as means ± S.E., $n = 4$.

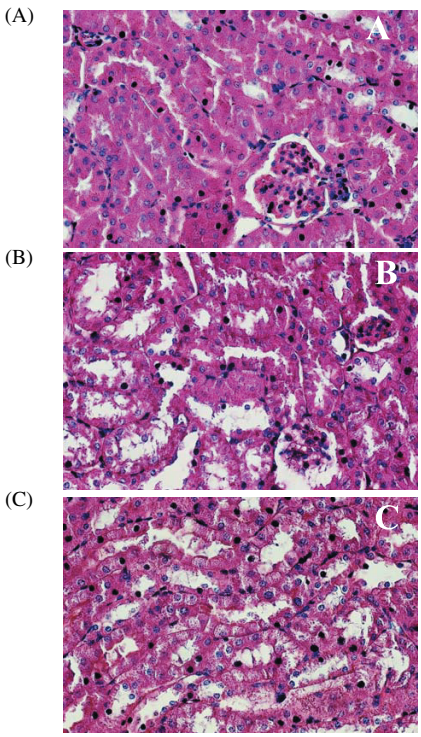

Fig. 7. Kidney Histopathology of *d*-Amphetamine-Treated Mice
(A) 5 mg/kg, with normal morphology. (B) 10 mg/kg, with some degree of cortical tubule degeneration. (C) 20 mg/kg, with an higher incidence of cortical tubule degeneration. Haematoxylin-eosin stain; original field magnification × 800.

DISCUSSION

Amphetamine is a drug of abuse consumed by a vast number of people, typically in a repeated manner, for high endurance performance tasks such as student examinations, long distance driving, factory

Table 1. Effect of (5, 10, 20 mg/kg, i.p.) *d*-Amphetamine on Kidney Histology

Treatment	Histological Findings	
	Tubular Degeneration	Haemorrhage Clots
5 mg/kg	—	—
10 mg/kg	+	+
20 mg/kg	+++	+

Extent of lesions in the kidney: (—) absence in all slides; (+) present in less than 25% of the slides; (+++) present in more than 75% of the slides.

work, warfare and rave parties, especially by young people. The consumption of this drug is illicit and controlled due to both the elicited behavioral deviations and the toxic effects reported in abusers, namely in the central nervous system and peripheral organs such as liver, kidney and heart.[11] Thus, there is a considerable clinical and forensic interest in the identification and quantification of these compounds in biological fluids.

In the present study, amphetamine excretion increased in the animals of the three groups in terms of mean values in a dose dependent manner, reaching fairly constant urinary levels after the third day. Taking into account its lipid-solubility, it is probable that the drug is accumulated in the organism due to partial reabsorption in the kidney and/or affinity to reservoir tissues. Considering the fairly steady state achieved thereafter, these biological mechanisms are probably saturated after the third day of repeated administration. Thus, amphetamine excretion seems to be a good and reliable qualitative and quantitative biomarker of amphetamine repeated administration to mice.

It has been questioned whether the toxic effects of amphetamine are only due to its indirect sympathomimetic activity or if there are other factors that can also play an important role.[5,12–15] Our group has shown the cytotoxic effects of *d*-amphetamine *in vitro* using isolated rat hepatocytes, and characterized the formation of a glutathione adduct of HA during the putative formation of an epoxide intermediate.[5] Thus, the measurement of urinary HA may be used as a biomarker of the reactivity of *d*-amphetamine that is non-related to its sympathomimetic effects. In the present study, the hepatotoxic effect was not evident in the histological analysis. However, the depletion of reduced glutathione may expose the liver to pro-oxidant deleterious conditions, like hyperthermia, as it was already demonstrated for 3,4-methylenedioxymethamphetamine (ecstasy).[16] The hydroxylation that occurs at either the *para*- or *meta*-position of the aromatic ring is favourably mediated by CYP2D because these positions are 5–7 Å of distance from the basic nitrogen of the amphetamines.[17] Five to 10% of Caucasians lack CYP2D6 activity because of the inheritance of two mutant CYP2D6 null alleles.[18] These subjects are classified as poor metabolizers (PM), with an impaired metabolism of CYP2D6 substrates. However, up to 7% of Caucasians and up to 29% of some African populations are ultrarapid metabolizers (UM) owing to the inheritance of alleles with duplication or amplification of functional CYP2D6 genes, causing an excessive amount of enzyme to be expressed.[18] These differences that are found among humans can be an explanation for the differences in toxic susceptibility that has often been reported.

Urinary excretion of HA increased in a time dependent manner. This could be explained by an induction of the aromatic hydroxylation enzymes. However, CYP2D6 is not inducible.[19] Thus, it is also possible that other inducible CYP isoenzymes may also contribute for amphetamine hydroxylation. Further studies are needed to corroborate this hypothesis. In a related study using Sprague-Dawley and Dark Agouti rats, this increase in HA excretion was not observed after the repeated administration of *d*-amphetamine (5 mg/kg, day, i.p.).[20] However, urine was only collected during 5 days, giving a similar excretion profile comparatively to the present study. The present findings indicate that longer-term studies are needed for a better understanding of the toxicokinetics of amphetamine.

Noteworthy, the urinary excretion of HA was dose independent during the whole time of *d*-amphetamine dosing. A possible explanation for this fact could be the saturation and/or the partial inhibition of the P450 enzymes involved in its aromatic hydroxylation. Importantly, an earlier report describing the effect of methamphetamine dose on urinary excretion of *p*-hydroxymethamphetamine noted that the excretion of the *p*-hydroxylated metabolite, as percentage of dose in 24 hr, was decreased with increasing dose (1, 5, 10, 20 and 45 mg/kg), while that of unchanged methamphetamine and amphetamine increased with increasing dose.[2] In that study, HA showed a similar excretion pro-

file, as compared to *p*-hydroxymethamphetamine. This effect is probably due to P450-mediated transformation of methamphetamine and amphetamine to their *N*-oxygenated metabolites, namely to *N*-hydroxymethamphetamine and *N*-hydroxyamphetamine, with subsequent inhibition of the enzyme system, as reviewed by Yamada *et al*.[2] Another factor contributing for this outcome could be also the observed amphetamine-induced nephrotoxic effects, with the consequent impairment of kidney function. Kidney damage has been reported as a consequence of human amphetamine abuse[7, 8, 21–23] and can be due to a combination of the direct renal effects of amphetamine and/or its metabolites, together with the amphetamine-induced rhabdomyolysis (and consequent deposit of the nephrotoxicant myoglobin in the kidney) hyperthermia and coagulopathy. The histological features of amphetamine-induced kidney damage in mice are shown for the first time in the present study, although an urinary biomarker of kidney toxicity (*N*-acetyl-β-D-glucosaminidase) was already shown to increase during amphetamine administration in rats.[13] It was also observed a decrease in body weight in a concentration and time dependent manner, likely due to *d*-amphetamine-induced toxicity. The decrease in body weight could otherwise be due to the anorectic effect of *d*-amphetamine but this effect was similar among the three groups and food intake was recovered from the 4th day.

The *d*-amphetamine sulfate dosage of 5, 10 and 20 mg/kg corresponds to 3.67, 7.34 and 14.68 mg/kg free base. For a 70 kg adult, this dosage would correspond to about 0.25, 0.5 and 1 g of amphetamine free base. Importantly, regular users of amphetamines may consume up to a few grams of the drug per day.[24–26]

In conclusion, the repeated administration of *d*-amphetamine at the dose of 10 mg/kg, day seems to be indicated for the study of amphetamine related effects in which the biotransformation into HA is involved. In fact, the repeated administration with this dose allowed the highest production of urinary HA, with toxic effects being already attained not only at the kidney level, but also observable by the decrease in body weight.

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