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Disposition of D-penicillamine, a promising drug for preventing alcohol-relapse. Influence of dose, chronic alcohol consumption and age: studies in rats

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ABSTRACT: Pharmacokinetic studies concerning D-penicillamine (an acetaldehyde sequestering agent) are scarce and have not evaluated the influence of chronic ethanol consumption and age on its disposition. Since recent preclinical studies propose D-penicillamine as a promising treatment for alcohol relapse, the main aim of the present work was to evaluate the influence of these two factors on D-penicillamine disposition in order to guide future clinical studies on the anti-relapse efficacy of this drug in alcoholism. Additionally, the effect of the administered dose was also evaluated. To this end, three studies were carried out. Study 1 assessed the influence of dose on D-penicillamine disposition, whereas studies 2 and 3 evaluated, respectively, the influence of chronic alcohol consumption and age. Rapid intravenous administrations of 2, 10 and 30 mg/kg of D-penicillamine were performed using young or adult ethanol-naïve rats or adult ethanol-experienced (subjected to a long-term ethanol self-administration protocol) rats. Pharmacokinetic parameters were derived from the biexponential model. Statistical analysis of *CL*, normalized AUC_0^∞, V_1 and k_{10} revealed that disposition, in the range plasma concentrations assayed, is non-linear both in young ethanol-naïve and in adult ethanol-experienced rats. Notably, no significant changes in $t_{1/2}$ were detected. Chronic ethanol consumption significantly reduced *CL* values by 35% without affecting $t_{1/2}$. D-Penicillamine disposition was equivalent in young and adult animals. In conclusion, although DP pharmacokinetics is non-linear, the lack of significant alterations of the $t_{1/2}$ would potentially simplify the clinical use of this drug. Chronic consumption of ethanol also alters D-penicillamine disposition but, again, does not modify $t_{1/2}$. Copyright © 2014 John Wiley & Sons, Ltd.

Key words: D-penicillamine; pharmacokinetics; chronic ethanol consumption

Introduction

D-Penicillamine (DP; (2S)-2-amino-3-methyl-3-sulfanylbutanoic acid) is a β-mercapto-α-amino acid used to treat several disorders such as Wilson's disease, hereditary cystinuria and rheumatoid arthritis refractory to conventional therapy [1–4]. In the 1970s/1980s, Nagasawa and colleagues [5–7] proposed the use of this and other similar amino acids (such as L-cysteine), which they named 'acetaldehyde sequestration agents', efficiently to inactivate under *in vivo* conditions acetaldehyde (ACD), the main metabolite of ethanol. The objective of their investigation was to develop useful medications able to prevent or limit the toxic effects associated with high levels of ACD in several population subgroups (for instance, Orientals lacking a functional ALDH2 or individuals treated with disulfiram or cyanamide) after chronic or abusive ethanol consumption. It should be kept in mind that ACD and its

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adducts with proteins and DNA constitute an important etiological factor in the induction of alcoholic liver disease [8–10].

In the past few years, a novel therapeutic indication for these acetaldehyde sequestration agents has been explored at a preclinical level: the prevention of relapse in alcoholism. The rational basis for this new indication lies in the discovery that acetaldehyde, locally generated in the brain after ethanol consumption, plays a crucial role in the excitation of dopamine neurons of the mesocorticolimbic system [11–16], a brain projection system critically involved in drug addiction [17–20]. The efficacy of D-penicillamine and L-cysteine to prevent activation of these dopamine neurons after ethanol or, even, exogenous acetaldehyde administration has been repeatedly demonstrated using different experimental paradigms [12,13,21–26]. Very recently, our group and others have shown that both D-penicillamine and L-cysteine efficiently prevent relapse in different animal models of alcoholism [23,25–27], reawakening interest in these compounds.

In spite of the broad experience in the clinical use of D-penicillamine in humans, several gaps in our knowledge should be filled before D-penicillamine is assayed in alcoholic patients to explore its anti-relapse efficacy in a clinical setup. For example, one important factor that needs to be explored is the influence that chronic consumption of ethanol could exert on DP pharmacokinetics. Chronic consumption of high doses of ethanol by alcoholic individuals provokes pathophysiological alterations in important body organs that could presumably alter D-penicillamine disposition. Moreover, the scarce pharmacokinetic literature available on animal models indicates that D-penicillamine disposition seems to be non-linear in a broad range of doses [28,29]. In fact, in our previous pre-clinical study on the anti-relapse efficacy of D-penicillamine a non-linear increase in D-penicillamine plasma levels was observed (by a factor of 6.5) when constant infusion rates increased four times, clearly suggesting that the disposition of D-penicillamine in our rat model of alcoholism was non-linear (see [26]). Thus it is necessary to assess whether D-penicillamine disposition depends on plasma levels of the drug using a concentration range including the effective plasma concentrations necessary for the anti-relapse effect and, if so, the pharmacokinetic parameters affected should be explored. Consequently, the present work evaluates the influence of dose and chronic alcohol consumption on D-penicillamine disposition in an adequate rat model.

Finally, since there is a non-negligible subgroup of elderly alcoholic patients who could benefit from this therapy; the influence of age on D-penicillamine pharmacokinetics after i.v. administration was also explored in our experimental rat model.

Material and Methods

Animals

Forty-three male Wistar rats were used in this study (from our own breeding colony at the Facultat de Farmàcia, Universitat de València) weighing 275 ± 10 g at the beginning of the experiment. All animals were individually housed in standard plastic cages ($42 \times 27 \times 18$ cm^3) with food and tap water provided *ad libitum* throughout the experimental period. Body weights were checked on a weekly basis. Artificial light was provided daily from 8:00 a.m. until 8:00 p.m., and room temperature and humidity were kept constant (temperature, 23 ± 1 °C; humidity, 60 ± 5%). Procedures were carried out in accordance with the EEC Council Directive 86/609, Spanish laws (RD 1201/2005) and animal protection policies. Experiments were approved previously by the Animal Care Committee of Universitat de València, Spain.

Drugs and chemicals

D-Penicillamine, obtained from Sigma-Aldrich Quimica, S.A. (Spain), was freshly dissolved in sterile saline at adequate concentrations (4, 20 or 60 mg/ml depending on the dose) for intravenous (i.v.) administration. Alcohol drinking solutions were prepared from 96% v/v (Scharlau S.A., Spain) and then diluted with tap water to the different concentrations tested.

All other dissolvents and reagents were of analytical or HPLC grade and were also purchased from Sigma-Aldrich Quimica, S.A. (Spain).

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Study 1. Influence of dose on D-penicillamine disposition: dose-linearity study

The influence of dose on D-penicillamine pharmacokinetics after i.v. administration was studied in two different subgroups of animals. The first group consisted of 21 young animals (9 weeks old) without any previous experience of ethanol consumption (young ethanol-naïve rats). The second group consisted of 18 adult rats (50–60 weeks old) subjected to a long-term ethanol self-administration protocol used in our recent preclinical study [26] (adult ethanol-experienced rats). Briefly, after 2 weeks of habituation to the animal room, animals belonging to the adult ethanol-experienced group were given continuous access to tap water and to 5%, 10% and 20% (v/v) ethanol solutions in their home cages. All drinking solutions were renewed weekly and, at that time, the animals were weighed and the positions of the four bottles were changed to avoid location preferences. Animals were subjected to five ethanol deprivation periods during which rats received only continuous access to tap water. The first 2-week deprivation period was introduced after 8 weeks of continuous ethanol availability. After this first deprivation period, the rats were given access to alcohol again, and then subjected to four additional deprivation periods in a random manner. Hence, the duration of the following drinking and deprivation periods was irregular: 6 ± 2 weeks and 2 ± 1 weeks, respectively, in order to prevent behavioral adaptations [30–32]. The animals showed a steady voluntary consumption, which was about 1 g/kg/day throughout the study (44 weeks).

Young animals were randomly assigned to one of the three experimental groups ($n = 7$). Then 24 h prior to the D-penicillamine administration, the rats were cannulated in the right jugular vein with a medical-grade silicone tube (Silastic, Dow Corning Co, Midland MI) [33]. Rats received 2, 10 or 30 mg/kg of D-penicillamine depending on the experimental group to which they had been assigned. Each dose was administered intravenously as a bolus in a 0.75 ml volume through the jugular vein cannula. Blood samples (0.2–0.3 ml) were drawn with the aid of heparinized syringes at the following times after dosing: 2, 5, 10, 15, 20, 40 and 60 min. For the 10 and 30 mg/kg doses an additional blood sample was drawn at 120 min. After each sampling, the blood volume was replaced with the same volume of sterile normal saline.

Adult animals with ethanol experience were also randomly assigned to one of three experimental groups ($n = 6$). As in the young animals, adult rats were also cannulated in the right jugular vein with a medical-grade silicone tube, 24 h prior to D-penicillamine administration. These adult rats also received 2, 10 or 30 mg/kg of DP, depending on the experimental group, using the same procedure for drug administration and blood sampling as in the young animals.

Study 2. Influence of chronic ethanol consumption on D-penicillamine disposition

Ten adult rats (50–60 weeks) were used to study the possible influence of chronic ethanol consumption on the disposition of D-penicillamine.

Data were derived, on the one hand, from the six adult rats subjected to the long-term ethanol self-administration protocol (see study 1) receiving the 10 mg/kg dose of D-penicillamine. These animals comprised the group named: adult ethanol-experienced group. On the other hand, four adult rats (group named: adult ethanol-naïve group) were given *ad libitum* access to tap water throughout the same period of time (i.e. a total of 44 weeks) as that used in the ethanol-experienced rats. These ethanol-naïve rats received the same surgical, drug administration and sampling procedures as the animals from the ethanol-experienced group. The pharmacokinetic data from this ethanol-naïve group served as a control group in this study and also in study 3 (see below).

Study 3. Influence of the age of animals on D-penicillamine disposition

To evaluate the influence of age on D-penicillamine disposition, the pharmacokinetic parameters derived from the young animals (9 weeks old) having received an i.v. bolus of 10 mg/kg of DP (see study 1) were compared with those obtained in adult rats (50–60 weeks old) belonging to the ethanol-naïve group (see study 2), also having received a bolus type i.v. injection of 10 mg/kg of D-penicillamine.

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Analytical methods

D-Penicillamine concentrations in plasma after i.v. bolus administration were evaluated from blood samples (0.2–0.3 ml) collected through the right jugular vein cannula at the specified sampling times with the aid of heparinized syringes. Blood samples were immediately centrifuged (1000 × *g* for 5 min) to obtain plasma. The D-penicillamine content in the samples was analysed by HPLC with electrochemical detection, as described previously [26]. As pointed out in that paper, one of the major difficulties in DP quantification is to maintain DP in its reduced state and to prevent its spontaneous oxidation in air to DP disulfide. Thus, biological samples must be treated immediately upon collection to stabilize the amount of DP present in the sample. This was basically accomplished by decreasing the pH in the sample with the so-called stabilizing solution (prepared by dissolving 4.39 g of diammonium hydrogen citrate and 100 g of metaphosphoric acid in 1 l of water). To quantify the DP plasma concentration, two previously reported protocols [29,34] were adapted to our experimental conditions. Briefly, 0.2 ml of the stabilizing solution was added to 0.1 ml of the plasma sample. The precipitated proteins were separated by centrifugation and the supernatant analysed.

The HPLC system consisted of an Alexis LC100 (Antec, Leyden, the Netherlands) pump in conjunction with an Intro (Antec) electrochemical detector. The applied potential was +0.8 V (ISAAC cell; Antec). The analytical samples and standards were injected onto a 2.6 µm C-18 column (Kinetex C-18; Phenomenex, Torrance, CA, USA) using a 5 µl loop valve. The mobile phase consisted of an aqueous solution, containing 0.116 g/l NaCl, 0.54 g/l 1-octanesulfonic acid and 4.52 g/l diammoniumhydrogen citrate, the pH being adjusted to 3.1. The mobile phase was pumped through the column at a flow rate of 0.1 ml/min. Chromatograms were integrated and compared with standards, which were freshly prepared and ran separately on each day of the experiment, using the AZUR 4.2 software (Datalys, France). The detection limit was defined by a signal-to-noise ratio of 2:1, which was approximately 4 ng/ml. The coefficient of variation of the method was below 5% in the 0.4–150 µg/ml range.

Pharmacokinetic methods and statistics

Plasma level curves of D-penicillamine after i.v. bolus administration were described by using the biexponential equation:

$$C = L_1 e^{-\lambda 1 t} + L_2 e^{-\lambda 2 t} \quad (1)$$

where C is the actual plasma level, L_i is the zero time intercept value for each disposition phase, and λ_i is the corresponding hybrid disposition rate constant ($\lambda_1 > \lambda_2$). The best estimates of these parameters were achieved through a weighted least-squares procedure, with the aid of a non-linear regression program [35]. This program uses the Marquardt-Levenberg algorithm to find the best estimates for the parameters of the equation. The weighting factor applied to the fit was $1/C^2$, the maximum number of iterations was fixed at 100 and the step size was set at $1 \cdot e^{-10}$. Once the best estimates of the parameters in Equation (1) were found, the following pharmacokinetic parameters were calculated using the recommended methods [36,37]: terminal disposition half-life, $t_{1/2}$, calculated as $0.693/\lambda_2$; the elimination rate constant, k_{10}, that was calculated as $\lambda_1 \cdot \lambda_2 \cdot (L_1 + L_2)/(\lambda_2 \cdot L_1 + \lambda_1 \cdot L_2)$ and the volume of distribution of the central compartment, V_1, was estimated as $D/(L_1 + L_2)$, D being the administered dose used. The total areas under plasma level curves, AUC_0^∞, were calculated by combining the areas from 0 to the respective last sampling time, estimated by the trapezoidal rule, with those obtained from this time point to infinity, calculated as the ratio C/λ_2, in which C is the corrected plasma level at the last sampling time [38]. Normalized AUC_0^∞ values with respect to the lower dose assayed (2 mg/kg) used in statistical comparisons in the dose linearity study (study 1), were calculated as the quotient:

$$\textit{Normalized } AUC_0^\infty = \frac{AUC_0^\infty \cdot 2(\text{mg/kg})}{D(\text{mg/kg})} \quad (2)$$

When required, the D-penicillamine plasma concentrations were also normalized (normalized concentration) applying the above equation, but using DP concentrations instead of AUC_0^∞.

The total plasma clearance, CL, was calculated as the ratio D/AUC_0^∞ [36,37]. When necessary, CL and V_1 were normalized by body weight (BW, in kg).

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Statistical comparisons of pharmacokinetic parameters obtained in study 1 were performed through a one-way analysis of variance (ANOVA) the administered dose being the between factor analysed. Two separate ANOVAs were performed, one for each experimental subgroup (young ethanol-naïve and adult ethanol-experienced rats). When significant differences were found in the ANOVA, the multiple comparison Tukey test was applied. Data from study 2 were compared using the Student's *t*-test for unpaired data. The same was done for the data in study 3. A probability level below 0.05 was considered to be statistically significant. Statistical analysis was performed using SPSS v15.0.

Results

Study 1. Influence of dose on D-penicillamine disposition: dose-linearity study

Mean plasma levels of D-penicillamine obtained in young rats without any experience of alcohol consumption after rapid i.v. administration of 2, 10 and 30 mg/kg of the drug are shown in Figure 1 (panel A). As can be seen in panel B of the figure, normalized plasma concentrations were not completely superimposable in this experimental group, suggesting the existence of non-linearities in DP disposition. The mean (± SD) L_1 and L_2 (in μg/ml) and λ_1 and λ_2 (in min^{-1}) values obtained after fitting Equation (1) to the DP plasma concentrations were as follows: 6.9 ± 1.9, 1.0 ± 0.2, 0.261 ± 0.018 and 0.025 ± 0.003 for the 2 mg/kg dose; 65.8 ± 46.7, 7.0 ± 3.2, 0.262 ± 0.160 and 0.023 ± 0.007 for the 10 mg/kg dose; 85.5 ± 7.4, 17.9 ± 8.4, 0.160 ± 0.046 and 0.022 ± 0.005 for the 30 mg/kg dose. Mean pharmacokinetic parameters derived from the above parameters are summarized in Table 1. Statistical comparisons revealed significant differences in *CL* ($p < 10^{-4}$), normalized AUC_0^∞ ($p < 10^{-4}$), V_1 ($p = 0.037$) and k_{10} ($p = 0.008$). Mean values of $t_{1/2}$ did not show any significant difference among them.

Figure 2 (panel A) illustrates the mean plasma level curves of DP after i.v. administration of 2, 10 and 30 mg/kg of the drug in adult rats with long experience of alcohol consumption. Normalized plasma concentrations observed in panel B of the figure were clearly not superimposable suggesting, again, a non-linear pharmacokinetic behavior of DP in the range of doses assayed. The mean (± SD) L_1 and L_2 (in μg/ml) and λ_1 and λ_2 (in min^{-1}) values obtained after fitting Equation (1) to DP plasma concentrations were as follows: 9.2 ± 3.2, 1.1 ± 0.5, 0.219 ± 0.075 and 0.024 ± 0.010 for the 2 mg/kg dose; 49.3 ± 11.2, 5.4 ± 3.0, 0.109 ± 0.026 and 0.015 ± 0.005 for the 10 mg/kg dose; 144.9 ± 18.5, 35.6 ± 6.8, 0.123 ± 0.019 and 0.016 ± 0.002 for the 30 mg/kg dose. Statistical analysis (Table 2) of the main pharmacokinetic parameters showed highly significant differences in *CL* ($p < 10^{-4}$) and normalized AUC_0^∞ ($p < 10^{-4}$). The mean k_{10} values also showed significant differences ($p < 10^{-4}$). No significant differences were found, either in V_1 or in $t_{1/2}$ values.

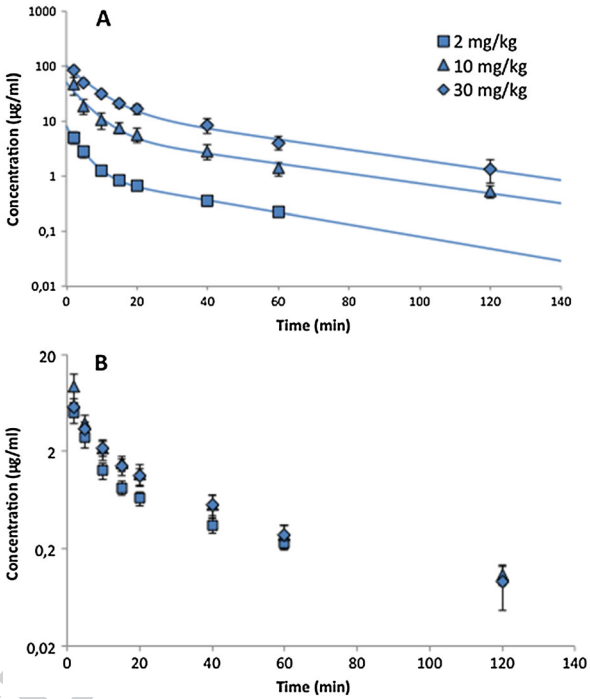

Figure 1. (A) Mean (± SD, $n = 7$) plasma concentration-time profiles of D-penicillamine after rapid i.v. administration of 2 (filled squares), 10 (filled triangles) and 30 (filled diamonds) mg/kg of the drug in young ethanol-naïve rats. Continuous lines represent theoretical plasma levels calculated with the respective fitted Equation (1). (B) Normalized mean (± SD, $n = 7$) plasma concentration–time profiles of D-penicillamine after i.v. bolus administration of 2 (filled squares), 10 (filled triangles) and 30 (filled diamonds) mg/kg of the drug in young ethanol-naïve rats. Values were normalized for the 2 mg/kg dose using Equation (2) (see text)

Table 1. Pharmacokinetic parameters of D-penicillamine after rapid i.v. administration of three different doses in young ethanol-naïve rats (mean ± SD, $n = 7$)

Dose (mg/kg)	CL (ml/min)	$t_{1/2}$ (min)	Normalized AUC_0^∞ (μg · min/ml)	V_1 (ml)	k_{10} (min^{-1})
2	10.8 ± 1.8^a	28 ± 4	66.9 ± 1^a	94 ± 25^{ab}	0.118 ± 0.013^a
10	6.3 ± 1.0^b	33 ± 10	$114.8 \pm$	66 ± 35^a	0.097 ± 0.032^{ab}
30	8.4 ± 1.7^b	33 ± 7	86.5 ± 19.3^c	110 ± 19^b	0.077 ± 0.009^b
Significance	$p = 0.004$	n.s.	$p < 10^{-4}$	$p = 0.037$	$p = 0.008$

[a,b,c] Groups with different superscripts showed significant differences in Tukey's test ($p < 0.05$).
n.s., denotes no significant differences in the ANOVA test at a 5% significance level.

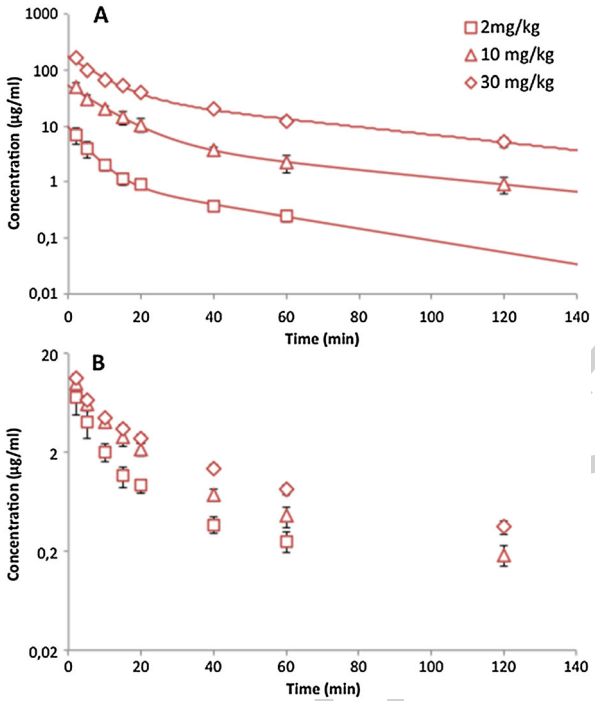

Figure 2. (A) Mean (± SD, $n = 6$) plasma concentration–time profiles of D-penicillamine after rapid i.v. administration of 2 (empty squares), 10 (empty triangles) and 30 (empty diamonds) mg/kg of the drug in adult ethanol-experienced rats. Continuous lines represent theoretical plasma levels calculated with the respective fitted Equation (1). (B) Normalized mean (± SD, $n = 6$) plasma concentration–time profiles of D-penicillamine after i.v. bolus administration of 2 (empty squares), 10 (empty triangles) and 30 (empty diamonds) mg/kg of the drug in adult ethanol-experienced rats. Values were normalized for the 2 mg/kg dose using Equation (2) (see text)

The mean AUC_0^∞ values plotted against the administered dose are shown in Figure 3 for both young and adult rats. As can be seen, the increase in the AUC_0^∞ values was not proportional (the dotted line in the figure illustrates the theoretical proportional increase) to the increase in the administered dose. Notably, this phenomenon was much more intense in adult rats with long experience of ethanol consumption.

Study 2. Influence of chronic ethanol consumption on D-penicillamine disposition

The effect of the chronic consumption of ethanol on D-penicillamine disposition after i.v. administration of 10 mg/kg of the drug was analysed in adult rats by comparing the main pharmacokinetic parameters derived from animals belonging to the ethanol-experienced and the ethanol-naïve groups. Figure 4 shows the mean plasma level curves compared in this study. The mean (± SD) L_1 and L_2 (in μg/ml) and λ_1 and λ_2 (in min^{-1}) values obtained after fitting Equation (1) to DP plasma concentrations were as follows: 49.3 ± 11.2, 5.4 ± 3.0, 0.109 ± 0.026 and 0.015 ± 0.005 for the ethanol-experienced rats and 38.4 ± 7.4, 3.8 ± 1.4, 0.148 ± 0.017 and 0.016 ± 0.005 for the ethanol-naïve rats. The derived pharmacokinetic parameters are summarized in Table 3. As can be observed, statistical comparisons revealed that CL values were significantly lower ($p = 0.011$) and AUC_0^∞ ($p = 0.02$) values significantly higher in ethanol-experienced rats. However, although the mean k_{10} and V_1 values tended to be higher in ethanol-naïve animals, statistical analysis did not show any significant difference between them. The mean $t_{1/2}$ values did not show any statistically significant difference. These data seem to indicate that the chronic consumption of ethanol provokes accumulative changes in the distribution and elimination processes of DP yielding to a significant change in the disposition of DP in rats.

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Table 2. Pharmacokinetic parameters of D-penicillamine after rapid i.v. administration of three different doses in adult ethanol-experienced rats (mean ± SD, $n = 6$)

Dose (mg/kg)	CL (ml/min)	$t_{1/2}$ (min)	Normalized AUC_0^∞ (μg·min/ml)	V_1 (ml)	k_{10} (min^{-1})
2	14.8 ± 4.5^a	35 ± 17	73.3 ± 22.4^a	138 ± 60	0.115 ± 0.031^a
10	7.3 ± 0.9^b	50 ± 16	138.7 ± 17.5^b	109 ± 20	0.069 ± 0.014^b
30	5.2 ± 0.4^b	44 ± 6	194.4 ± 13.7	98 ± 16	0.053 ± 0.007^b
Significance	$p < 10^{-4}$	n.s.	$p < 10^{-4}$	n.s.	$p < 10^{-4}$

[a,b,c]Groups with different superscripts showed significant differences in Tukey's test ($p < 0.05$) .
n.s., denotes no significant differences in the ANOVA test at a 5% significance level.

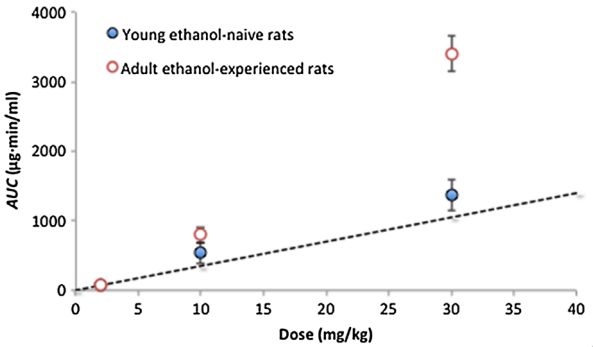

Figure 3. Plot of the AUC_0^∞ values against the administered dose of D-penicillamine. Data from study 1 using young ethanol-naïve (blue-filled circles) and adult ethanol-experienced (red empty circles) rats. The theoretical proportional increase of AUC_0^∞ values with the dose is illustrated in the figure with the dotted line

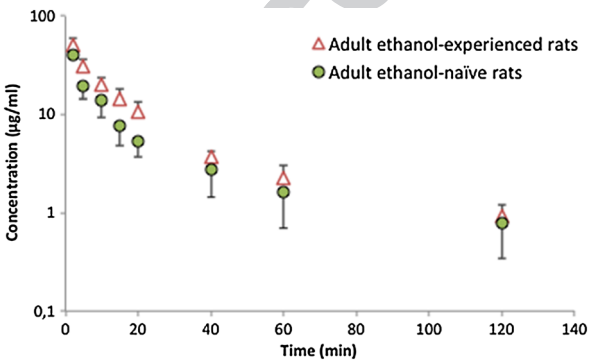

Figure 4. Mean (± SD) plasma concentration–time profiles of D-penicillamine after rapid iv administration of 10 mg/kg of the drug in adult ethanol-experienced (red empty triangles) ($n = 7$) and adult ethanol-naïve (green-filled circles) rats ($n = 4$)

Study 3. Influence of the age of animals on D-penicillamine disposition

The analysis of the possible influence of age on DP disposition was accomplished by comparing the pharmacokinetic parameters obtained after i.v. administration of 10 mg/kg of the drug to young and adult animals without any prior experience of ethanol consumption. Figure 5 shows the mean plasma level curves compared in this study. Table 4 summarizes the main results of this study. The mean CL values showed significant differences between young and adult rats ($p = 0.002$). Since the great difference ($p = 0.001$) in the mean BW values between the adult and young rats could explain the observed difference in CL, this parameter was re-evaluated after normalization of CL values by body weight. The new variable, CL/BW, did not show significant differences between the groups. The same occurred with V_1 values. Moreover, no significant differences were found for the remaining pharmacokinetic parameters analysed. In conclusion, age did not seem to influence the disposition of D-penicillamine in rats.

Discussion

The present study shows that D-penicillamine disposition is clearly non-linear in the range of doses assayed (2–30 mg/kg) both in young animals without any prior experience with alcohol and in adult rats with a long experience of alcohol consumption. Moreover, chronic voluntary ingestion of alcohol provoked a significant reduction in the total clearance of D-penicillamine in adult rats. These results should have a significant influence on the design of future studies on the clinical

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Table 3. Pharmacokinetic parameters of D-penicillamine after rapid i.v. administration (10 mg/kg) in adult ethanol-experienced and ethanol-naïve rats (mean ± SD)

Experimental group	Body weight (kg)	*CL* (ml/min)	*CL*/BW (ml/min/kg)	$t_{1/2}$ (min)	Normalized AUC_0^∞ (μg·min/ml)	V_1 (ml)	k_{10} (min^{-1})
Ethanol-experienced	0.57 ± 0.09	7.3 ± 0.9	12.9 ± 1.8	50 ± 16	798.1 ± 117.5	109 ± 20	0.069 ± 0.014
Ethanol-naïve	0.53 ± 0.09	11.0 ± 2.6	20.6 ± 3.0	45 ± 14	492.5 ± 70.1	128 ± 19	0.086 ± 0.017
Significance	n.s.	$p = 0.011$	$p = 0.001$	n.s.	$p = 0.02$	n.s.	n.s.

n.s., denotes no significant differences in the t-test at a 5% significance level.
BW, body weight.

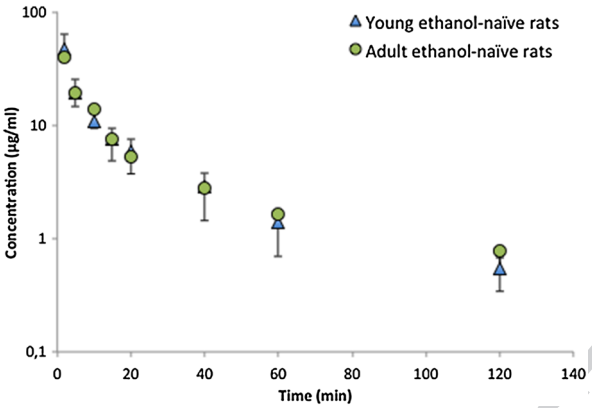

Figure 5. Mean (± SD) plasma concentration–time profiles of D-penicillamine after rapid i.v. administration of 10 mg/kg of the drug in young (blue-filled triangles) ($n = 7$) and adult (green-filled circles) ethanol-naïve rats ($n = 4$)

applicability of this sequestration agent in the treatment of alcohol relapse.

Studies on DP pharmacokinetics are scarce. As noted by Bergstrom and colleagues, this paucity could be due to the lack of a specific and sensitive assay for the compound [29]. The present study used a recently developed analytical procedure of HPLC with electrochemical detection with adequate specificity and sensitivity to conduct a pharmacokinetic study on the disposition of unchanged D-penicillamine, allowing DP plasma levels to be measured that were one order of magnitude below the effective concentration for the anti-relapse effect. Our analytical method employed a procedure, also reported previously by Bergstrom *et al*. [29], to stabilize the reduced DP present in plasma samples. In our previous paper, by using this method conveniently adapted to our experimental conditions, we were able to determine the effective concentration of DP in plasma to prevent alcohol relapse in our rat model of alcoholism [26]. This concentration was approximately 3–4 μg/ml in plasma. It was also shown that D-penicillamine efficiently crosses the blood–brain barrier reaching its biophase, probably located in the ventral tegmental area of the rat brain. Moreover, brain DP concentrations were about 2–4% of the corresponding plasma concentrations [26].

The present results clearly indicate that DP pharmacokinetics after rapid i.v. administration is non-linear. The *CL* values were reduced by 20–40% in young animals and 50–65% in adult animals when the DP dose increased from 2 mg/kg to 30 mg/kg. Within this range of doses, measured DP plasma levels oscillated between 100 μg/ml and 0.4 μg/ml. Since in our previous paper, the

Table 4. Pharmacokinetic parameters of D-penicillamine after rapid i.v. administration (10 mg/kg) in young and adult ethanol-naïve rats (mean ± SD)

Experimental group	Body weight (kg)	*CL* (ml/min)	*CL*/BW (ml/min/kg)	$t_{1/2}$ (min)	Normalized AUC_0^∞ (μg·min/ml)	V_1 (ml)	V_1/BW (ml/kg)	k_{10} (min^{-1})
Young	0.32 ± 0.04	6.3 ± 1.0	19.8 ± 4.8	33 ± 10	537.0 ± 151.8	66 ± 35	211 ± 123	0.097 ± 0.032
Adult	0.53 ± 0.09	11.0 ± 2.6	20.6 ± 3.0	45 ± 14	492.5 ± 70.1	128 ± 19	245 ± 56	0.069 ± 0.014
Significance	$p = 0.001$	$p = 0.002$	n.s.	n.s.	n.s.	$p = 0.014$	n.s.	n.s.

n.s., denotes no significant differences in the *t*-test at a 5% significance level.
BW, body weight.

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effective concentration was 3–4 μg/ml, the present results clearly indicate that DP pharmacokinetics will be non-linear in the range of the therapeutic concentrations, which is an important finding that should be taken into account in the future design of therapies using D-penicillamine to prevent relapse.

Another remarkable and useful finding for the future clinical management of DP in the prevention of relapse in alcoholism is related to the small value of the terminal half-life of DP. D-Penicillamine plasma concentrations fall rapidly after i.v. administration in rats. This phenomenon also occurs in dogs [29] and in humans after i.v. administration [39,40]. The same has been observed after oral administration of the drug. For example, [41] reported $t_{1/2}$ values between 1.37 and 3.15 h in human volunteers after oral administration of 250 to 1000 mg. Similar values were reported by other authors [39,42,43]. Importantly, previous reports in humans have shown that $t_{1/2}$ of DP seems to be dose-independent [43]. The present results are in close agreement with those findings. The mean $t_{1/2}$ values did not change significantly with the increase of the administered dose. The mean $t_{1/2}$ values oscillated between 28 and 50 min and, moreover, neither depended on age nor ethanol experience of the animals. Theoretically, these findings simplify the design of multiple dosage regimens since the $t_{1/2}$ is the only variable that determines the rate at which a drug accumulates in the body during regular multiple dosing. However, the small $t_{1/2}$ value could constitute an obstacle in the application of conventional pharmaceutical dosage forms when D-penicillamine would be administered in multiple dosage regimens (it would require very short dosing intervals). The use of prolonged or controlled drug delivery systems could be an optimal alternative. In fact, in our previous study evaluating the minimum effective concentration for the anti-relapse effect of D-penicillamine, we used mini-osmotic pumps to deliver continuously (zero order kinetics) D-penicillamine into the subcutaneous tissue [26], thus circumventing the problem derived from the short $t_{1/2}$.

In addition, another objective of the present study was to determine whether a long history of chronic alcohol consumption may affect the pharmacokinetics of D-penicillamine. This was a relevant objective, as our research is designed to incorporate D-penicillamine into the therapeutic arsenal useful to prevent relapse in alcoholic patients. It is noteworthy that the potential recipients for this new indication of D-penicillamine would be alcoholics with a long history of excessive alcohol consumption but, in a voluntary abstinence phase at the time of receiving D-penicillamine treatment. The ethanol deprivation protocol used in our experiments is able to reproduce these special abstinence conditions of potential patients to be treated with D-penicillamine. Although it is fundamental to validate the anti-relapse efficacy of DP, this protocol would seem to be unwarranted for the analysis of the influence of ethanol on DP disposition given the huge cost in time and labor required for its implementation. Nonetheless, bearing in mind our final goal and the adequate number of animals subjected to this long-term protocol in our laboratory, finally it was decided to use this protocol in spite of the existence of other simpler and less expensive experimental models of alcoholism.

Our present data reveal that a prolonged history of alcohol consumption reduces the *CL* of D-penicillamine in rats. The reduction in the mean *CL* values obtained in ethanol-experienced rats was about 35% relative to those of adult rats without alcohol experience. Importantly, this significant reduction in clearance was not accompanied by significant changes in $t_{1/2}$. The causes for this reduction in *CL* are not known at present. Whether a reduced or altered hepatic function or another ethanol-induced pathophysiological change are responsible for our results is not yet known. The k_{10} values tended to diminish slightly in rats with previous alcohol experience, although there were no significant differences with respect to ethanol-naïve animals. A similar trend was observed for the mean V_1 values, and, again, no significant differences were found. A combination of altered elimination and distribution processes is likely to explain the significant reduction observed in the *CL* values. Whether this finding could be of importance in humans is presently unknown. However, the lack of a significant change in $t_{1/2}$ values as a consequence of chronic alcohol consumption would simplify the clinical use of DP in alcoholic individuals. Finally, the age of the animals (when the effect of body weight was

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discounted) did not condition the pharmacokinetics of D-penicillamine. Both CL/BW and $t_{1/2}$ were independent of the age of the animals. Assuming that the pharmacokinetics of D-penicillamine is similar in rats and humans, the present finding suggests that the clinical use of D-penicillamine in elderly patients should not be significantly different to that in young individuals, although more experiments are required to confirm this assertion.

Conclusions

Our findings indicate that D-penicillamine pharmacokinetics is non-linear in a range of doses comprising the one likely to be applied in therapy for its anti-relapse effect, although the lack of significant alterations of the half-life would potentially simplify the clinical use of this drug. The chronic consumption of ethanol also alters D-penicillamine disposition but, again, does not modify the terminal half-life of the drug. Finally, D-penicillamine pharmacokinetics is not altered in aged rats.

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Conflict of Interest

Q2 The authors report no conflict of interest.

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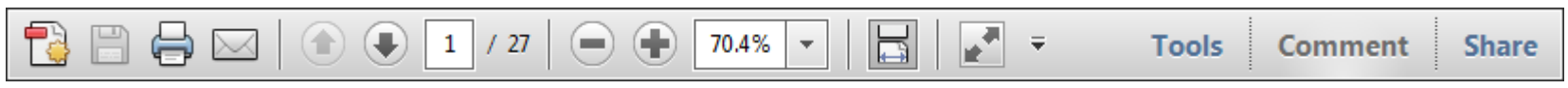

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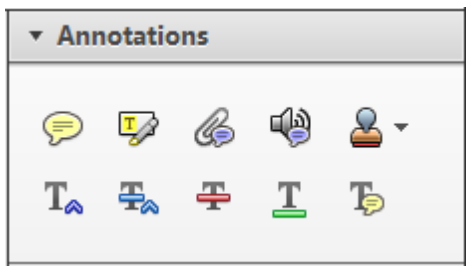

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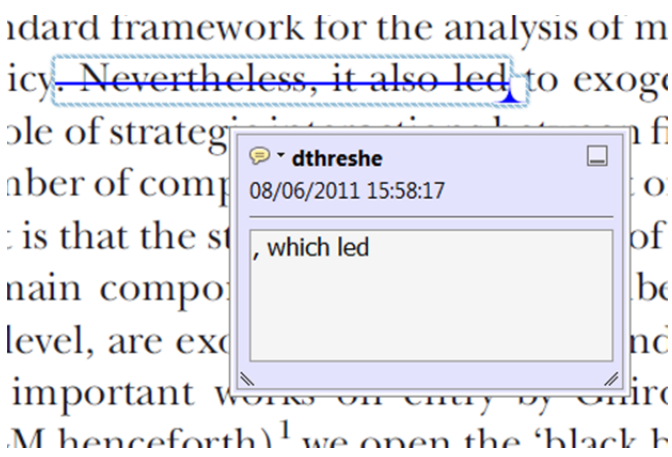

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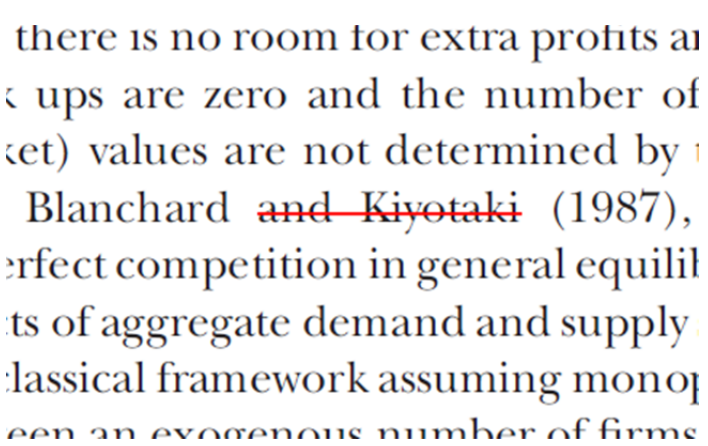

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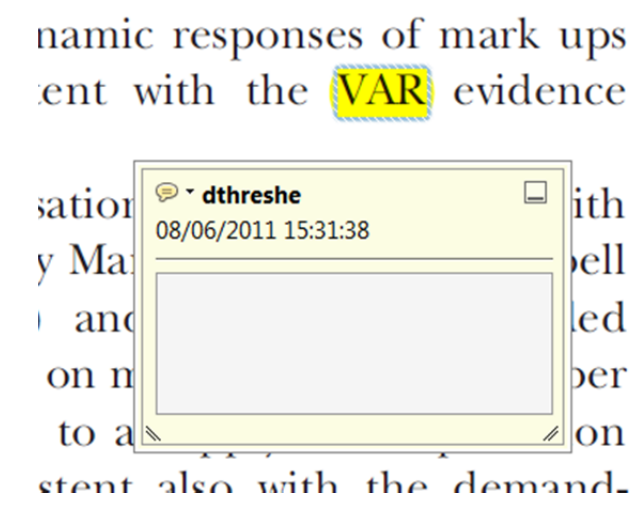

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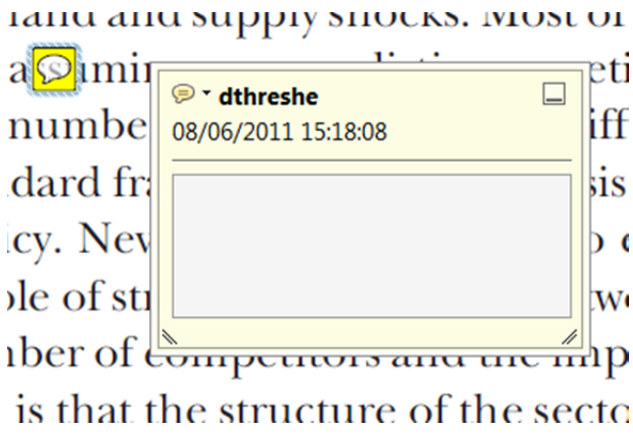

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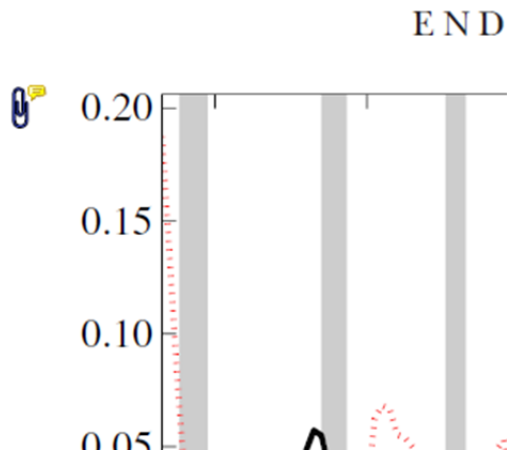

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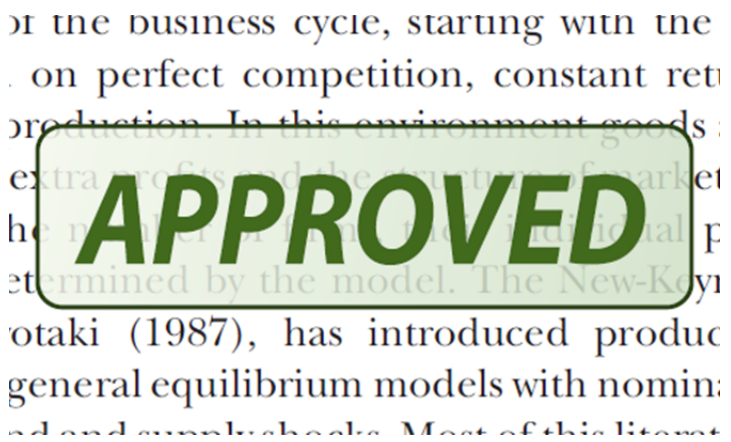

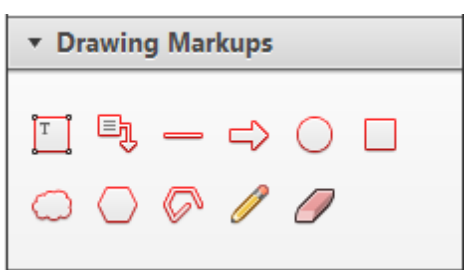

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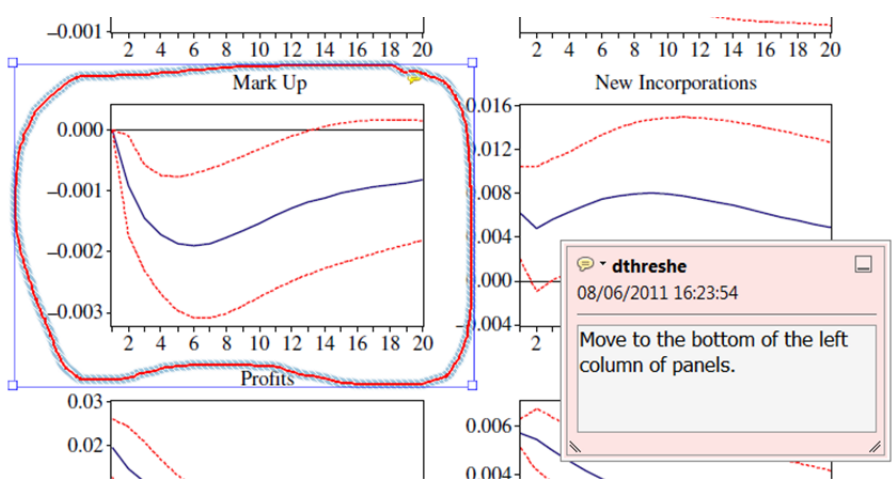

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