Screening of the inhibitory effect of xenobiotics on alcohol metabolism using S9 rat liver fractions

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A B S T R A C T

The purpose of this work was to develop and optimize a simple and suitable method to detect the potential inhibitory effect of drugs and medicines on alcohol dehydrogenase (ADH) activity in order to evaluate the possible interactions between medicines and alcohol metabolism. Commonly used medicines that are often involved in court litigations related with driving under the influence of alcohol were selected. Alprazolam, flunitrazepam and tramadol were tested as drugs with no known effect on ADH activity. Cimetidine, reported previously as having inhibitory effect on ADH, and 4-methylpyrazole (4-MP), a well known ADH inhibitor, were tested as positive controls. Apart from 4-MP, tramadol was identified as having the higher inhibitory effect with an IC50 of 44.7 × 10^{-3} mM, followed by cimetidine (IC50 of 122.9 × 10^{-3} mM). Alprazolam and flunitrazepam also reduced liver ADH activity but to a smaller extent (inhibition of 11.8 ± 5.0% for alprazolam 1.0 mM and 34.5 ± 7.1% for flunitrazepam 0.04 mM). Apart from cimetidine, this is the first report describing the inhibitory effect of these drugs on ethanol metabolism. The results also show the suitability of the method to screen for inhibitory effect of drugs on ethanol metabolism helping to identify drugs for which further study is justified.

1. Introduction

It is well documented that the administration of drugs and/or medicines together with concomitant alcohol (ethanol) consumption may lead to toxic interactions or liver damage. Some of these interactions are attributable to pharmacokinetic changes caused by alcohol in the hepatic drug metabolism pathways catalyzed by the various CYP enzymes (e.g. Tanaka et al., 2007; Weathermon and Crabb, 1999 and others cited therein). What is not so well known is the potential effect of drugs and medicines on the metabolic clearance of ethanol, whose initial step is driven by alcohol dehydrogenase (ADH), the major alcohol-metabolizing enzyme. In fact, most studies in the literature are designed to detect the effects caused by alcohol upon the metabolism of drugs and other xenobiotics but not the reverse. Nevertheless, the knowledge of interactions between xenobiotics and ethanol metabolism would be crucial because if a drug significantly inhibits liver ADH, it will prevent ethanol metabolic clearance at normal rates and thus lead to a concomitant increase of blood alcohol concentration (BAC).

According to an epidemiological study conducted in 13 European countries under the DRUID project (Driving Under Influence of Alcohol Drugs and Medicines), ethanol was the most common psychoactive substance in all age groups among seriously injured drivers admitted in emergency departments. The mean and median values of blood alcohol concentration (BAC) were 1.59 g/L and 1.60 g/L, respectively. In cases of polysubstance use, alcohol was also the main substance associated with benzodiazepines and/or illicit drugs (DRUID, 2011a). As many of those drivers are probably heavy drinkers, it will be important to adopt prevention protocols as proposed by Cunningham et al. (2009). Given the epidemiology of polysubstance use, patients, health professionals and regulatory bodies should be aware that such drugs present an increased risk of slowing down alcohol metabolism, which may result in potentially toxic plasma alcohol concentrations and the related alcohol–health consequences, causing associate risk behavioral impairment along with psychosocial, legal and forensic implications (Cunningham et al., 2009; DRUID, 2011a,b,c; EMCCDA, 2008).

Given the current lack of available data, a routine method to screen for ADH inhibition by drugs would clearly be very useful; however, a standardized method that allows this is not available. As we are interested in study these potential interactions and in order to fill this gap, a spectrophotometric method based on the
procedure described by Kägi and Vallee (1960) was adapted and optimized. As a source of ADH, rat liver homogenates S9 fraction was used as it is easily available and is rich in the main ethanol-metabolizing enzyme ADH1. Moreover, rat and human ADH1 are phylogenetically related (Holmes, 2009; Höög et al., 2001), exhibiting many kinetic analogies (Julià et al., 1987), thus supporting the reason why the rat has been the animal most used in pharmacological and metabolic studies of ethanol metabolism (Julià et al., 1987).

In order to test the method and evaluate the possible interactions between medicines and alcohol metabolism, and based on information from the Department of Toxicology of the National Institute of Legal Medicine – South Branch (Lisbon, Portugal) we selected some commonly used/abused substances that are often involved in court litigations related to driving under the influence of alcohol. Cimetidine, a histamine H2–receptor antagonist used to treat peptic ulcers, was chosen because it is a known inhibitor of gastric and hepatic ADH (Battiston et al., 1995; Dawidek-Pietryka et al., 1998; Mirmiran-Yazdy et al., 1995; Pozzato et al., 1992; Stone et al., 1995). Alprazolam, used to treat moderate to severe anxiety disorders, and flunitrazepam, used as antianxiety and hypnotic sedative, were selected because toxic interactions with ethanol or drug-related deaths have already been described with these two benzodiazepines (Michaud et al., 1999; Tanaka, 2002; Tanaka et al., 2005, 2007). Tramadol, a centrally-acting analgesic used against moderate to severe pain, was chosen because there are published cases of tramadol involvement in drug-related deaths and impairment (Michaud et al., 1999; Moore et al., 1999) but no data on the interaction of this compound with the metabolism of ethanol is available.

2. Materials and methods

2.1. Chemicals

Nicotinamidé adenine dinucleotide (β-NAD), sodium pyrophosphate decahydrate, dimethylsulfoxide 99.9% (DMSO) and phosphate-buffered saline pH 7.4 (PBS) were obtained from Sigma, absolute ethanol was obtained from Panreac, and 4-methylpyrazole-HCl (4-MP) was purchased from Aldrich. Alprazolam, cimetidine, flunitrazepam and tramadol, were kindly provided by the Department of Toxicology of the National Institute of Legal Medicine – South Branch (Lisbon, Portugal). All other chemicals and reagents used were of analytical grade.

Stock solutions of NAD+ (15.0 mM), sodium pyrophosphate buffer (50.0 mM, pH 8.8 at 25 °C), absolute ethanol diluted to 95% (v/v), 4-MP (1.0 mM), and tramadol–HCl (10.0 mM) were prepared in recently deionised water and stored in aliquots at −20 °C, to be used when needed. Alprazolam, cimetidine and flunitrazepam do not have adequate water solubility and were dissolved in DMSO up to their solubility limit. In these cases, extemporaneous solutions of progressively lower concentrations were prepared until no precipitation occurred in the UV cuvette.

2.2. Preparation of S9 rat liver fraction

Freshly excised livers from three adult Wistar rats were freed from fat and connective tissue and immersed in ice-cold PBS. The inner tissue was weighed, placed in a small beaker, minced and homogenized in PBS (ratio 1:3, w/v) kept on ice during the procedure. The homogenates were obtained using a Potter–Elvehjem homogenizer and centrifuged at 9000g for 20 min at 4 °C in an Eppendorf 5810R centrifuge equipped with a F45–30–11 Rotor. The obtained supernatants (fraction S9) were immediately aliquoted and stored frozen at −80 °C until used. The protein concentration in the S9 fraction (4.3 mg/ml) was determined according to the Bradford technique (Bradford, 1976) using bovine serum albumin as the standard.

2.3. Alcohol dehydrogenase activity assays

Spectrophotometric measurements were performed at 340 nm and at 25 °C in a Shimadzu UV-1601 spectrophotometer. Sodium pyrophosphate buffer, NAD+ and drug solutions were prewarmed to 25 °C. An aliquot of the S9 liver fraction was defrosted and kept on ice during the experiments. The assays were performed in a final volume of 1 ml in a 1 cm optical path quartz cuvette containing sodium pyrophosphate buffer (22 mM), S9 liver fraction (143 µg total protein), and NAD+ solution (7.5 mM). An auto-zero at 340 nm was performed and the changes in absorbance registered during 4 min (blank without substrate), corresponding to a steady increase in the optical density due to a non-specific NADH formation. Then, ethanol 95% (final concentration 3.2% v/v; 550 mM) was rapidly added and mixed, and the absorbance recorded each second for another 4 min. When xenobiotics were tested, the volume of pyrophosphate buffer was reduced to keep the same final volume in the cuvette. Alprazolam (1.0 mM), cimetidine (0.1 × 10−3 to 10.0 mM), flunitrazepam (0.04 mM), tramadol (0.1 × 10−3 to 10.0 mM) and 4-MP (0.01 × 10−3 to 1.0 mM) were added prior to the addition of NAD+ and substrate. A proper control with DMSO (1% v/v final concentration) was done to exclude the effect of this solvent.

ADH specific activity was calculated from the differences in absorbance before and after the addition of ethanol during 4 min (ΔA150/min, see Fig. 1). NADH concentration was calculated using 6.22 as the millimolar extinction coefficient of β-NADH at 340 nm, and normalized to the protein used in the assay (mU/mg protein). The known inhibitor of ethanol metabolism, 4-MP, was used as a positive control.

Dose–response curves were performed for the compounds which solubility allowed to obtain concentrations with a maximum observable effect (cimetidine, tramadol and 4-MP). The concentration for 50% inhibition (IC50) was calculated by nonlinear regression of dose–response curves (GraphPad Prism, Inc.) to the equation

\[ y = y_{\text{min}} + \left( y_{\text{max}} - y_{\text{min}} \right) \left( 1 + 10^{(\log IC50 - \text{Inh})} \right) \]

where \( y \) is the percentage inhibition obtained for each concentration of inhibitor [Inh] and \( H \) is the Hill coefficient.

2.4. Statistical analysis

Data were presented as mean ± standard error of the mean (SEM) of at least four independent experiments for each of the tested compounds, and were analyzed with Student’s t test. A value of \( P \leq 0.05 \) was considered to be statistically significant.

3. Results and discussion

A literature search was undertaken to investigate the possible involvement of some medicines and drugs on the ADH-catalyzed oxidation of alcohol. A few studies were performed using ADH from sources as diverse as isolated human hepatocytes (Cornell et al., 1983), hepatocyte 100,000 fractions (Gergel and Cederbaum, 1996; Pozzato et al., 1992), horse liver (Bychkov et al., 2005), or baker’s yeast (Jin et al., 2004) but no systematic screening of the inhibition of alcohol metabolism by drugs and other xenobiotics was ever performed. A suitable and standardized screening procedure is also not available. For these reasons, a spectrophotometric enzymatic assay of ADH activity (Kagi and Vallee, 1960) was adapted and optimized to monitor its inhibition in a rat liver homogenate. Rat liver homogenate S9 fraction was chosen for developing and optimizing a screening procedure, since it is easily
available. Although the rat as a whole is not the best model to study alcohol metabolism because the rat ADH2 almost lacks ethanol oxidizing capacity, the rat liver is rich in ADH1, and this is the main ethanol metabolizing enzyme, showing a good homology with the human ADH1 pool (Höög et al., 2001).

Preliminary experiments were carried out in whole liver homogenates diluted to different protein concentrations and with different substrate (ethanol) concentrations (not shown). However, this medium proved unsuitable, as the background “noise” at 340 nm was too high preventing absorbance readings. The S9 fraction was therefore tested as it contains both cytosol (where the ADH enzymes are found) and microsomes. The results showed that the S9 fraction provides a much smaller noise allowing good absorbance readings. The use of the soluble fraction alone (100,000 g supernatant) was not considered as it is more difficult to prepare and does not bring additional advantage.

Fig. 1 shows a typical spectrophotometric measurement of the rat liver ADH activity obtained with the S9 fraction. It can be seen that after ethanol addition to the cuvette, the increase in absorbance due to NADH production can be used to determine the initial rate of the catalyzed reaction. In the presence of one of the xenobiotics tested (here flunitrazepam 0.04 mM) the initial rate was clearly reduced evidencing a decrease in ADH activity.

To ensure that ADH activity was being accurately assessed with this technique, experiments were carried out in the absence (100% activity, 45.1 mU/mg protein) and in the presence of different concentrations of a well-known specific ADH inhibitor, 4-MP (0.01 × 10⁻³ to 1.0 mM). As expected, 4-MP showed a concentration-dependent inhibition of ethanol dehydrogenation. At the highest concentration tested (1.0 mM) 4-MP inhibited ADH by 93.1 ± 4.6%, reaching a residual activity of 3.1 mU/mg protein. By performing a dose–response curve, an IC50 of 3.58 × 10⁻³ mM was calculated. At the highest concentration tested (10.0 mM) cimetidine inhibited ADH by 51.0 ± 3.0%. These results are consistent with previous studies using human purified and recombinant ADH where an inhibitory kinetic profile was characterized for different gastric and liver ADH isoenzymes (Stone et al., 1995). Moreover, a similar ADH inhibitory effect of cimetidine on rat gastric epithelial cells, which ranged from 27.4% (0.1 mM) to 38.9% (1.0 mM), was also reported (Mirmiran-Yazdy et al., 1995).

Alprazolam and flunitrazepam also showed an inhibitory effect on liver ADH activity (11.8 ± 5.0% for 1.0 mM alprazolam and 34.5 ± 7.1% for 0.04 mM flunitrazepam, respectively) (Fig. 2). However, since these substances were tested at their maximum solubility concentration, it was not possible to measure their effect at concentrations significantly higher to obtain a dose–response curve. Although toxic interactions with ethanol have already been described for both compounds (Michaud et al., 1999; Tanaka et al., 2005, 2007), there are no published data of a metabolic interaction between these drugs and ethanol. In fact, and as far as we know, this is the first time that the inhibitory effect of alprazolam and flunitrazepam on ethanol metabolism is described. This information may be important given the high prevalence of psychoactive drugs use in the general population and among drivers (DRUID 2011a,b,c; EMCDDA, 2008).

Tramadol (0.1 × 10⁻³ to 10.0 mM) was also found to markedly inhibit ADH activity. The calculated IC50 was 44.7 × 10⁻³ mM, a lower value than the obtained for cimetidine, therefore pointing to a higher inhibitory potency of this drug. Although there are

**Fig. 1.** Typical spectrophotometric traces of rat liver alcohol dehydrogenase (ADH) activity. The formation of NADH at 340 nm in the absence (control) and in the presence of flunitrazepam (0.04 mM) shows a reduced ADH activity by the drug. The arrow indicates the addition of ethanol to the media triggering NADH production.

**Fig. 2.** ADH activity in the presence of alprazolam and flunitrazepam. The presence of alprazolam (1.0 mM) and flunitrazepam (0.04 mM) in the reaction media induced a decrease in ADH activity (45.1, 39.8 and 29.5 mU/mg prot for control, alprazolam and flunitrazepam, respectively). Results are expressed as percentage of control and as mean ± SEM of six independent experiments. *P < 0.05, **P < 0.0001.
published cases of tramadol involvement in drug-related deaths and impairment (Michaud et al., 1999; Moore et al., 1999), no data on the interaction of this compound with the metabolism of ethanol is available. As with flunitrazepam and alprazolam and to the best of our knowledge, this is the first report on the inhibitory action of this drug on ethanol metabolism.

4. Conclusion

The present work presents a suitable method to screen for inhibition of liver ADH, thus allowing to detect the inhibitory effects of drugs on alcohol metabolism. The procedure uses rat liver S9 fraction, an easily obtained fraction rich in the main ethanol metabolizing enzyme ADH1, and was validated using 4-MP, a known inhibitor of ADH activity, and cimetidine a drug already reported as an ADH inhibitor. This procedure seems therefore suitable for screening a large number of compounds that otherwise wouldn’t be possible using human samples.

The results obtained using this procedure, not only emphasize the potential inhibitory effect of cimetidine on liver ADH but also unravel for the first time that alprazolam, flunitrazepam and tramadol, three commonly prescribed medicines, inhibit to different extents the in vitro rat liver ADH activity which may correlate to alcohol incidents and multi-risk behaviors impact. Tramadol was identified as having an inhibitory effect higher than cimetidine showing that this compound certainly deserves further study in human samples. The small inhibition observed with the benzodiazepines suggests that the pharmacodynamic interaction between alcohol and these compounds are probably not primarily due to the in vivo inhibition of alcohol metabolism.

Taking advantage of the screening technique developed, additional work should be carried out to evaluate the potential effect of other xenobiotics on ethanol metabolism. Moreover, these results shed some light on which drugs deserve to be further tested using human samples.

References