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RESEARCH ARTICLE

Purification and characterization of dihydropyrimidine dehydrogenase enzyme from sheep liver and determination of the effects of some anaesthetic and antidepressant drugs on the enzyme activity

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Abstract

Dihydropyrimidine dehydrogenase (DPD, E.C. 1.3.1.2) was purified from sheep liver with a yield of 16.7%, purification fold of 407.5 and specific activity of 0.705 EU/mg proteins. The purification procedure consisted of ammonium sulphate fractionation, DEAE ion exchange chromatography and 2′,5′-ADP Sepharose-4B affinity chromatography. The molecular weight determined by SDS-PAGE and was found 111 kDa. Optimum pH, ionic strength temperature and stable pH were determined as 8.0, 0.9 mM, 50 °C and 6.0, respectively. The kinetic parameters (K_m and V_{max}) of the enzyme were determined with NADPH as 22.97 μM and 0.17 EU/mL, respectively. The same parameters were determined with uracil as 17.46 μM and 0.14 EU/mL, respectively. Additionally, *in vitro* inhibitory effects of some antidepressant drugs including escitalopram, fluoxetine, mirtazapine, haloperidol and some anaesthetic drugs including propofol and lidocaine were investigated against DPD. In addition, IC_{50} values for each active drug obtained for escitalopram, fluoxetine, mirtazapine, haloperidol, propofol and lidocaine were determined as 1736.11, 13.24, 86.65, 99.03, 0.21 and 15.07 μM, respectively.

Keywords

Anaesthetic agent, antidepressant drug, dihydropyrimidine dehydrogenase, enzyme inhibition, enzyme purification

History

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Introduction

Dihydropyrimidine dehydrogenase (DPD) is the first and rate-limiting enzyme in pyrimidine catabolism. DPD catalyses the NADPH-dependent reduction of uracil and thymine to 5,6-dihydrouracil and 5,6-dihydrothymine, respectively[1]. In addition, DPD degrades 5,6-florouracil, fluorinated pyrimidine to 5,6-dihydrofluorouracil. 5-Florourasil (5FU) is one of the most commonly used anticancer drugs to treat colorectal, breast, head and neck cancers. Anti-tumour and cytotoxic effects are produced by inclusion of the remaining 5FU into anabolic pathways through inhibition of thymidylate synthase, which is a required enzyme for the *de novo* synthesis of deoxythymidine monophosphate (dTMP). Further enzymatic activity is limited to RNA and DNA synthesis and stability are decreased as a result of transformation of 5FU to deoxyuridine monophosphate (5FdUMP), which comprises a stable complex with thymidylate synthase[2].

High sequence similarity (>90%) between human and other mammalian species, such as bovine and pig has a similar reaction mechanism and three-dimensional structure[3]. Up to now, DPD was purified from a variety of species such as rat[4], pig[5], human[6], cow[7], cynomolgus monkey, rhesus monkey, dog, mouse liver[8] and *Alcaligenes eutrophus*[9]. Molecular masses of DPD from human, pig, rat, cow liver and *Alcaligenes eutrophus* were found as 210, 204, 210, 216 and 52 kDa, respectively[7,9]. Recently, pharmacokinetic studies have mainly focused on DPD, which is the key enzyme of 5-FU catabolism. Today, numerous clinical reports show that totally abolished capacity for patients to detoxify 5-FU, has a dramatic effect on drug disposition. It is now fully acknowledged that DPD deficiency is responsible for most potentially lethal toxicities in patients to whom the drug has been administered at standard dosages. Additionally, drug interactions and circadian variations are non-genetic causes regularly evoked to explain the large interpatient variability observed in DPD's activity[10,11].

A complete DPD deficiency causes growth retardation, dysmorphic features, microcephaly and motor retardation[12]. The association is indicated in patients with partial or complete DPD activity profound side effects, including mucositis, granulocytopenia, neuropathy and even death after the administration of 5FU[13].

DPD activity is determined in sheep liver and *in vitro* effects of escitalopram, mirtazapine, fluoxetine, haloperidol, propofol and lidocaine on sheep liver DPD were investigated. Cancer patients can have depression throughout diagnosis and treatment of their disease. While some of the patients show transient symptoms, a number of the other patients manifest significant depressive symptoms that affect their physical, emotional and social functioning. The increment of the quality of life is important and affects cancer treatment[13]. Escitalopram, mirtazapine, fluoxetine and haloperidol are antidepressant drugs. Escitalopram, the

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S-enantiomer of citalopram, and fluoxetine are selective serotonin reuptake inhibitor antidepressants[14]. Mirtazapine is used for the enhancement of noradrenergic and specific serotonergic systems in central nervous system[15]. Haloperidol is an antipsychotic drug for the treatment of acute and chronic schizophrenia and other disorders worldwide[16]. The effects of anaesthetic drugs have been studied *in vitro* and in animal models and some human studies. Propofol has a lot of biological effects and is a short acting general anaesthetic drug and sometimes accepted as a sedative agent[17–23]. Propofol conjugates have been indicated to inhibit cellular adhesion, migration and apoptosis in breast cancer cells[24]. Lidocaine is a short-acting local and regional anaesthetic and antiarrhythmic agent[25]. In addition, it has been observed to inhibit tumour cells proliferation[24]. A comprehensive study addressing the purification of DPD from sheep liver is not met in the literature.

The aim of this study was to purify DPD from sheep liver and to determine its kinetic values and parameters. Also, another goal of this research was to determine the effects of some anaesthetic and antidepressant drugs including *in vitro* effects of escitalopram, mirtazapine, fluoxetine, haloperidol, propofol and lidocaine on sheep liver DPD.

Experimental

Purification of DPD

The procedure of purification of DPD enzyme's procedure was based on Shiotoni and Weber[4] and Podschun et al.[5] with slight modification.

Crude extract

Fifteen grams of sheep liver was minced and cleaved with liquid nitrogen. Then it was homogenized in 25 mL buffer A containing 35 mM potassium phosphate (pH 7.4, 2.5 mM $MgCI_2$), aminoethyl isothiouronium bromide (1 mM), sucrose (0.25 M), EDTA (10 mM), dithioerythritol (2 mM) and 2-mercaptoethanol (5 mM) using a ultraturrax. This homogenate was centrifuged at 27 000 × *g* for 30 min followed by an additional centrifugation at 100 000 × *g* for 60 min.

Ammonium sulphate fractionation

Ammonium sulphate [$(NH_4)_2SO_4$] precipitation method was described previously[26]. Solid $(NH_4)_2SO_4$ was slowly added to the supernatant of the second centrifugation until a 30% saturation obtained. The mixture was stirred for 30 min and then centrifuged at 45 000 × *g* for 15 min. $(NH_4)_2SO_4$ was added to the supernatant until 50% saturation was obtained with constant stirring for 30 min. The enzyme solution was centrifuged and the 30–50% $(NH_4)_2SO_4$ precipitate was dissolved in buffer A containing 0.5 mM dithioerythritol and dialyzed against the same buffer for 1 h. Then, pH of the $(NH_4)_2SO_4$ fraction was adjusted to 4.85 with 5% acetic acid. The solution was stirred for 10 min at this pH (4.85) and then centrifuged for 10 min at 43 500 × *g* to remove the precipitated protein. The supernatant was readjusted to pH 7.3 with solid Tris and dialyzed against buffer A plus 0.5 mM dithioerythritol.

DEAE-cellulose chromatography

After dialysis, the enzyme solution was centrifuged at 43 500 × *g* for 10 min and applied to DEAE-cellulose column chromatography, equilibrated with buffer A plus 0.5 mM dithioerythritol. The column was washed with the same buffer. Thirty-five millimolar KCI in buffer A plus 0.5 mM dithioerythritol was used to elute the enzyme.

2′,5′-ADP-Sepharose-4B column chromatography

After dialysis against 1 L buffer A plus 0.5 mM dithioerythritol, the enzyme solution was applied to a 2′,5′-ADP-Sepharose-4B column chromatography. The affinity column had been washed with equilibration buffer until all unbound protein was eluted completely. The enzyme was eluted with 0.2 mM NADPH and 1 M KCI in buffer A. Enzyme fractions (1.5 mL) were collected and dialyzed with 1 L buffer A plus 0.5 mM dithioerythritol.

Determination of DPD activity

DPD activity was measured at 25 °C (room temperature) according to Podschun et al.[5] One mL of total reaction volume contained phosphate buffer (35 mM, pH 7.4), $MgCl_2 \cdot 6H_2O$ (280 mM), dithioerythritol (20 mM), NADPH (600 μM) and uracil (1500 μM). The activity was measured by monitoring the decrease in absorbance at 340 nm due to oxidation of NADPH to $NADP^+$. One enzyme unit is defined as the oxidation of 1 mmol NADPH per min under the assay conditions.

Protein determination

Quantitative protein determination was measured spectrophotometrically at 595 nm with bovine serum albumin being used as a standard[27–32].

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of the purified enzyme was performed according to Laemmli's method[33] described previously[21,34–39]. It was carried out in 3% and 10% acrylamide concentration for stacking and running gel, respectively, containing 0.1% SDS (Figure 2). After electrophoresis was completed, the protein bands were stained with silver staining[40].

Optimum pH determination

A buffer system between pH 5.0 and 10.0 was used to determine the pH at which enzyme activity was the greatest. In order to determine the optimal pH, enzyme activities were assessed using 280 mM phosphate buffer with pH ranging between 5.0 and 8.0, and 280 mM Glycine–NaOH buffer with pH ranging between 8.5 and 10.0. DPD enzyme activity was measured spectrophotometrically in this buffering range according to the procedure described for the DPD activity assay.

Ionic strength determination

In order to determine optimal ionic strength of sheep liver DPD enzyme, after the assessment of optimal pH, activity measurements were performed at optimal pH using 1.0, 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2 and 0.1 mM phosphate buffers.

Optimum temperature determination

A digital water bath was used to determine the optimum temperature for enzyme activity between 0 and 80 °C. To determine the optimal temperature for DPD enzyme purified from sheep liver, activity measurements were performed at optimal pH and ionic strength while increasing the temperature in steps of 10 °C between 0 and 80 °C.

Stable pH determination

In order to determine stable pH of sheep liver DPD enzyme, 35 mM KH_2PO_4 buffers at pH 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0 were used. An aliquot of the buffer solutions (1 mL) at the specified pH values was mixed with 1 mL of enzyme solution and

then it was stored at +4 °C. The stable pH values obtained from this assay were used for all the other experiments.

Kinetic studies

K_m and V_{max} values for NADPH and uracil substrates were calculated from Lineweaver–Burk curves[41]. DPD activities were determined while keeping one of the substrates constant and varying the other substrate at five different concentrations. Using the obtained $1/V$ and $1/[S]$ values, Lineweaver–Burk graphics were plotted and K_m and V_{max} values were determined[42–51].

Inhibition studies

To determine the effects of antidepressant drugs including escitalopram, fluoxetine, mirtazapine, haloperidol antidepressant drugs and anaesthetic drugs including propofol and lidocaine on sheep liver DPD activity, activity measurements were performed with the addition of five different concentrations of these drugs were added into the measurement cuvette. DPD activity was measured and control cuvette activity in the absence of drug was taken as 100%[52–55]. For each antidepressant and anaesthetic drugs an Activity (%)–[Drug] graph was drawn and IC_{50} values were obtained from this graph[50,51].

Results and discussion

Pyrimidines are key monomers of nucleic acids. Selective inhibition of their catabolic metabolism is important for the design of antitumor, antimicrobial and antiparasitic agents[5]. DPD is the first and also the rate-limiting enzyme catalysing the breakdown of uracil and thymine to β-alanine and β-aminoisobutyrate, respectively; this is the only pathway for β-alanine synthesis in mammals. DPD activity is present in many tissues, predominantly in liver and peripheral mononuclear blood cells. DPD is also responsible for the metabolism of more than 80% of 5-fluorouracil (5-FU), which is an anti-cancer agent used in the treatment of breast, head/neck and colorectal cancers. 5-FU is generally well tolerated; however, patients who have low or undetectable DPD activity are prone to severe and even life threatening toxic conditions such as pancytopenia, mucositis and neurological toxicity. DPD deficiency is autosomal recessive. Complete DPD deficiency results in congenital thymine–uracil syndrome, characterized with increases in urinary thymine and uracil, and this condition causes growth retardation and convulsive disorders[8]. In DPD deficient patients, 5FU administration results in toxic conditions that may be fatal. Some studies have shown that molecular defects in dihydropyrimidine gene (DPYD) result in insufficient activity of DPD, which leads to pharmacogenetic syndrome. Today, there are over 40 reported variant alleles including several mutations on DPYD coding region. Measurement of DPD activity is necessary to have a clear association between the phenotype and genotype of this pharmacogenetic syndrome[52].

The kinetic parameters, K_m and V_{max} were determined using NADPH and uracil as co-substrates. K_m values were calculated as 22.97 and 17.46 μM, V_{max} values were found as 0.17 and 1.14 EU/mL for NADPH and uracil, respectively (Figure 1). Podschun et al. reported K_m values in DPD purified from porcine liver as 1.98 μM and 11.36 μM for uracil and NADPH substrates, respectively[5]; Shiotani and Weber reported K_m values in DPD enzyme purified from rat as 1.8 μM and 11.00 μM and V_{max} values as 41.5 EU/mL and 40.6 EU/mL for uracil and NADPH substrates, respectively[4]. Lu et al. reported K_m values of DPD enzymes purified from pig, rat and cow liver as 4.9, 5.9, 2.4 and 3.9 μM, respectively[6]. V_{max} values as 0.6 (μmol mg^{-1}), 0.7 (μmol mg^{-1}), 0.8 (μmol mg^{-1}), 0.7 (μmol mg^{-1}), respectively

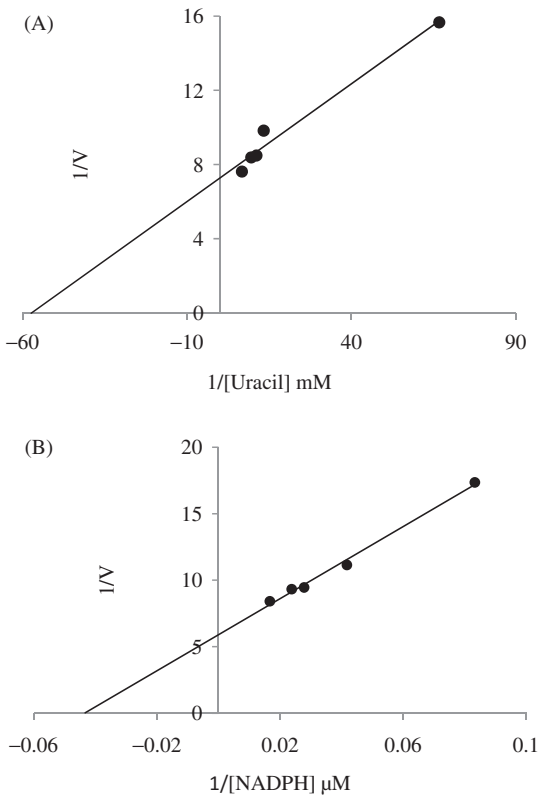

Figure 1. (A) Lineweaver–Burk graph of five different uracil concentrations and in constant NADPH concentration. (B) Lineweaver–Burk graph of five different NADPH concentrations and in constant uracil concentration.

for uracil substrate; for NADPH substrate, they reported K_m values of DPD enzyme purified from pig, rat and cow liver as 9.6, 11.9, 20.6 and 8.9 μM, respectively, and V_{max} values as 10.1 (μmol mg^{-1}), 8.2 (μmol mg^{-1}), 20.9 (μmol mg^{-1}), 8.5 (μmol mg^{-1}), respectively[6]. It is observed that K_m values of sheep liver DPD for NADPH substrate are close to the results in literature compared to K_m values for uracil substrate. Additionally, V_{max} values for NADPH overlap with the results in literature more than V_{max} values for uracil. In line with the results in literature, K_m value for uracil is smaller than K_m value for NADPH. Lesser K_m values indicate solidity of enzyme–substrate complex, while higher K_m values show that the affinity of the enzyme to the substrate is less.

DPD enzyme has been purified from sheep liver for the first time in this study. For this purpose, DEAE-anion exchange chromatography and affinity chromatography were used in the first place. Then, kinetic properties of the purified enzymes were determined and molecular weight was assessed with SDS-PAGE method. The obtained homogenate was precipitated with solid $(NH_4)_2SO_4$ first and then it was precipitated with acetic acid precipitation at pH 4.85. Quantitative protein measurement was done in eluates obtained with DEAE ion exchange chromatography first and then in eluates obtained with 2′,5′-ADP Sepharose-4B affinity chromatography. Protein quantitation in eluates was made according to Bradford method. In this method, Coomassie Brilliant Blue G-250, which has a negative charge, binds to positively charged regions in proteins, and the resulting coloured complex has a maximum absorbance at 595 nm. The upside of this method that makes it superior to other protein analysis methods is that it is less sensitive to interfering effects and has high precision. Binding of dye to protein takes place very rapidly and the protein–dye complex remains in solution for a long time. Sensitivity of this method is between 1 and 100 mg[53].

Table 1. Summary of purification steps of dihydropyrimidine dehydrogenase (DPD) from sheep liver.

Purification steps	Volume (mL)	Enzyme activity (EU/mL)	Protein (mg/mL)	Total activity (EU)	Total protein (mg)	Specific activity (EU/mg)	Purification fold	Yield (%)
Crude homogenate	27.0	0.073	42.00	1.971	1134.0	1.73×10^{-3}	1.0	100
Ammonium sulphate precipitation	14.0	0.055	29.65	0.770	415.1	1.85×10^{-3}	1.07	39.1
DEAE cellulose ion exchange chromatography	4.5	0.028	0.078	0.126	0.351	0.359	207.5	6.4
2′,5′-ADP-sepharose-4B affinity chromatography	1.0	0.012	0.017	0.012	0.017	0.705	407.5	6.1

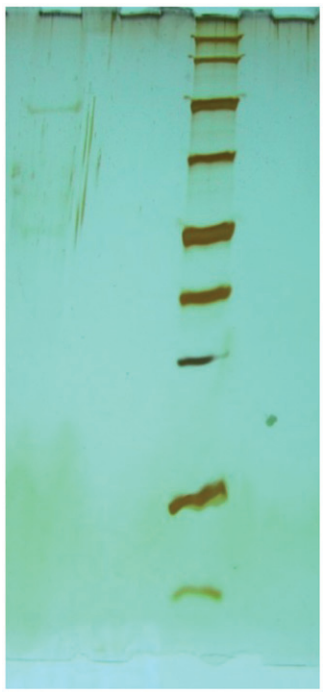

Figure 2. SDS-PAGE bands of DPD enzyme from sheep liver. Gel stained using the silver staining procedure. Lane 1: affinity chromatograph; lane 2: standard proteins (SDS-PAGE: sodium dodecyl sulphate polyacrylamide gel electrophoresis, DPD: dihydropyrimidine dehydrogenase enzyme).

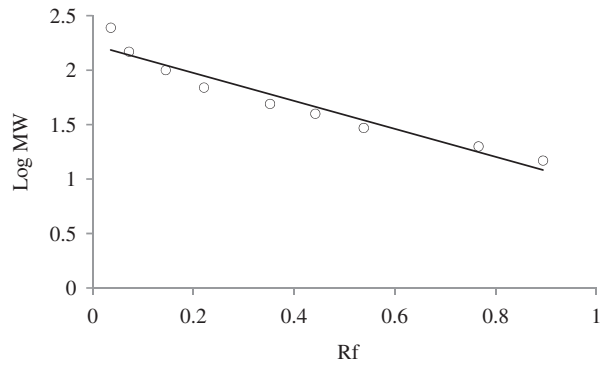

Figure 3. Standard R_f-Log MW graph for the determination of molecular weight of sheep liver DPD.

During preparation of sheep liver homogenate, liquid nitrogen and ultraturax were used for the purpose of cellular lysis. After ammonium sulphate precipitation and dialysis, the homogenate was injected to DEAE-cellulose column and affinity column. DPD enzyme was purified from sheep liver using 30–50% saturated ammonium sulphate fraction, treatment at pH 4.85, DEAE ion exchange chromatography, 2′,5′-ADP-Sepharose-4B affinity chromatography (Table 1). As a result of the study, DPD was purified 407.5-fold with a yield of 6.1% and a specific activity of 0.705 EU/mg protein (Table 1). Activity measurement and quantitative protein measurement were carried out in obtained eluates[54–64]. According to studies in the literature, it has been purified to 3100-fold with 28% in pig[5], to 1406-fold with 15% efficiency in rat[4]; and in studies using HPLC, it has been purified to 7824-fold with 20.3% efficiency in human, to 2588-fold with 16.7% efficiency in pig, to 3700-fold with 42.7% efficiency in rat and 3669-fold with 30.6% efficiency in cow[6].

The purification of DPD was determined by SDS-PAGE and exhibited a band, as shown in Figure 2. As can be seen in the figure, a band is observed for DPD after silver staining. Some proteins with molecular weights of 250, 150, 100, 70, 50, 40, 30, 20 and 15 kDa were used as standards. Moreover, as can be seen in column 1, R_f values were calculated for standard proteins and DPD. R_f–Log MW graph was obtained according to Laemmli's procedure[33]. Molecular weight of DPD was calculated as 111 kDa (Figure 3). It was reported that molecular masses of pig were found as three bands (12, 92 and 107 kDa) using affinity column[5]. On the other hand, it was found 110 kDa for rat[4]. When HPLC was used for purification, molecular mass of DPD was determined as 102 and 90 kDa as two bands in human and pig. Similarly, molecular mass of this enzyme was 105 kDa in rat and 108 kDa in cow[5]. Our molecular mass result for DPD is in coherence with the results in literature.

After the purification processes, characterization studies were carried out for the enzyme. For this purpose, optimal pH, optimal ionic strength, optimal temperature and stable pH values were determined for sheep liver DPD enzyme. Optimal pH value for sheep liver DPD enzyme was determined as 8.0 in 0.28 M KH_2PO_4 buffer (Figure 4A). Enzyme activities were measured from 0.01 to 1.0 M phosphate so as to specify the effects of ionic strength on the enzyme activity. Maximum enzyme activity was obtained in 0.28 M phosphate buffer (pH 8.0). Therefore, the optimum ionic strength of enzyme is 0.9 M (Figure 4B). Enzyme activities were measured between 0 and 80 °C in order to determine the temperature profile of DPD (Figure 4C). Enzyme activity increased after 0 °C. Also, the maximum activity was observed at 50 °C. Therefore, the optimum temperature of enzyme is 50 °C (Figure 4C). According to activity measurements at 12 h intervals for the following four days, the pH value at which the enzyme is stable was determined as 6.0 (Figure 4D). It was previously reported that optimal pH values of DPD enzyme was 7.4 in phosphate buffer for rat[4], 7.4 in phosphate buffer for human and 7.4 in cow[6]. According to activity measurements ionic strength for the enzyme was determined as 0.9 M (Figure 4) and optimal temperature was determined as 50 °C (Figure 4C). The optimal temperature was reported as 37 °C in liver DPD enzyme purified from human[6].

In this study, inhibition effects of escitalopram, fluoxetine, mirtazapine, haloperidol, propofol and lidocaine drugs on sheep liver DPD were investigated and IC_{50} values were calculated. IC_{50} values were determined from Activity (%)–(Drugs) graph[65–71] (Table 2). IC_{50} values for escitalopram, fluoxetine, mirtazapine,

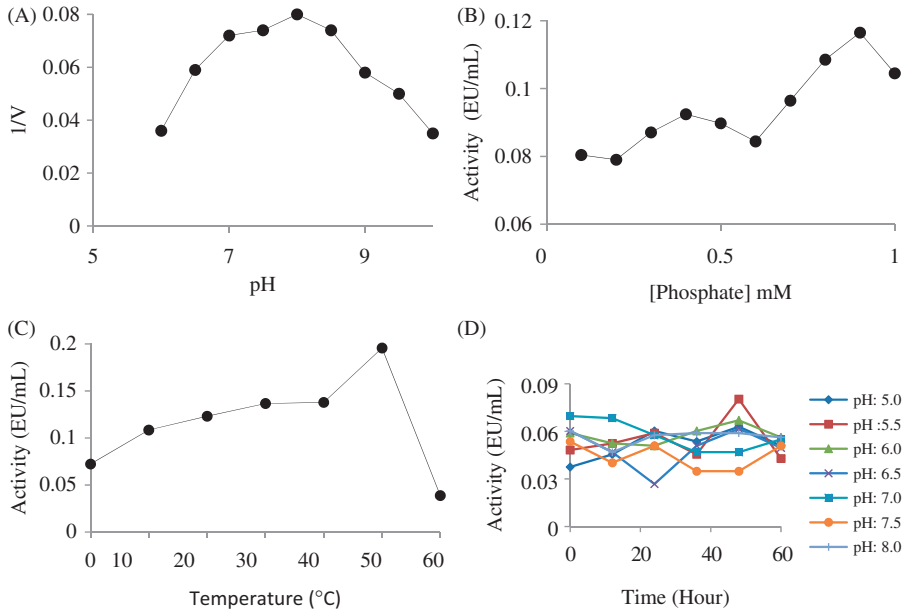

Figure 4. (A) The effect of pH on the activity of sheep liver DPD. (B) The effect of ionic strength on the activity of sheep liver DPD. (C) The effect of temperature on the activity of sheep liver DPD. (D) Determination of stable pH for sheep liver DPD.

Table 2. The half maximal inhibitory concentration (IC_{50}) of some antidepressant and anaesthetic drugs against dihydropyrimidine dehydrogenase (DPD) from sheep liver.

Drugs	IC_{50} values (μM)
Escitalopram	1736.11
Fluoxetine	13.24
Mirtazapine	86.65
Haloperidol	99.03
Propofol	0.21
Lidocaine	15.07

haloperidol, propofol and lidocaine were 1736.11, 13.24, 86.65, 99.03, 0.21 and 15.07 μM, respectively (Table 2). The results clearly showed the propofol had powerful inhibition effect. It has been referred to as milk of amnesia, because of the milk-like appearance of its intravenous preparation[72]. Breakdown of 5FU drug results in many fluorinated products, which cause toxicity in the organism. DPD is necessary to break down and eliminate these products. Inhibition of DPD is a desirable situation in cancer patients, since this leads to increased sensitivity to 5FU drug. However, excess inhibition, or complete or partial deficiency of DPD in some patients may cause severe toxicity. DPD deficiency also causes neurological deficits.

There are a few studies related to drug inhibition of DPD enzyme activity. Therefore, the results that we obtained on this enzyme have not been reported before. It is determined that due to the inhibition of DPD enzyme, administration and dose adjustment of drugs containing such active ingredient is of significance.

Conclusion

The effect of some antidepressant and anaesthetic drugs against DPD from sheep liver was evaluated, in the present study. DPD was effectively inhibited by both chemical drug groups with IC_{50} values in the range of 0.21–1736.11 μM. Especially, propofol as intravenously administered amnestic agent strongly inhibited DPD with Ki of 0.21 nM. Propofol has been frequently used for the induction and maintenance of general anaesthesia, sedation for mechanically ventilated adults and procedural sedation. Propofol is also commonly used in veterinary medicine. For this reason, propofol must be carefully used and their dosages should be very well ordered to decrease the side effects.

Declaration of interest

The authors have declared no conflict of interest.

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