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RESEARCH ARTICLE

In vitro efficacy of some cattle drugs on bovine serum paraoxonase 1 (PON1) activity

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Abstract

Serum paraoxonase 1 (EC 3.1.8.1, PON1), a calcium-associated enzyme, has an ability to hydrolyze organophosphate compounds. Related to this property, PON1 has a critical role in antioxidant mechanisms. It is well-known that the enzyme protects LDL from oxidation. In this study we investigated the *in vitro* inhibitory effects of some drugs. These drugs are oxytocin, dexamethasone, atropine sulphate, gentamicin sulphate, sulfadoxine-trimethoprim, furosemid, metamizole sodium and toldimfos sodium. The IC_{50} values obtained varied markedly from 0.014 to 507.72 mg/mL. According to our findings, most potent and significant inhibition was displayed by dexamethasone, atropine sulphate and furosemid.

Keywords: Paraoxonase, inhibition, oxytocin, dexamethasone, atropine sulphate, gentamicin sulphate, sulfadoxine-trimethoprim, furosemid, metamizole sodium, toldimfos sodium

Introduction

The serum A-esterase PON1, which is primarily synthesized in liver and secreted into blood, catalyses the hydrolysis of organophosphates (OP) that have been used in production of insecticides and neurotoxic gases, and thus the enzyme has great importance for xenobiotic metabolism *in vivo* and toxicological studies[1].

PON1 plays an important physiological role in lipid metabolism by hydrolysis of oxidized lipids, in the form of lipid hydroperoxides, generated on lipoproteins such as *High-density lipoprotein* (HDL) and *Low-density lipoprotein* (LDL) and within this ability it is considered as an antioxidative/anti-inflammatory component of HDL. Thus it provides protection against the development of oxidative stress, though the exact mechanism by which PON1 reverse and prevent liver damage by exogenous antioxidants PON1 enzyme protects LDL, bad cholesterol, from oxidation and neutralizes radicals including hydrogen peroxide by its antioxidant property[2]. It also hydrolyzes oxidized cholesteryl linoleate hydroperoxides in LDL and specific oxidized phospholipids[3]. According to its antioxidant role, PON1 concentration was also found to be inversely correlated with the development of atherosclerosis, and its reduced activity is associated with hypercholesterolemia, diabetes, and coronary vascular disease (CVD)[4–6]. Decreased activity of PON1 has been postulated as a risk factor for CVD[4–6].

Mitochondria are the important sites for production of reactive oxygen species (ROS) due to incomplete reduction of molecular oxygen to water as a consequence of electron leakage in the electron transport chain[7,8]. Increased oxygen consumption and oxidative phosphorylation result in increased production of ROS[9,10]. Ohara *et al.* reported that higher levels of plasma lipids are associated with increased production of ROS[11].

The oxygen analogs of a number of OPs (e.g. paraoxon, chlorpyrifos oxon, diazinon oxon) are hydrolyzed by PON1, which plays a central role in their detoxication and toxicity[12]. While PON1 activity can be detected at low levels in most tissues, very high levels are present in rat liver and plasma, with plasma accounting for more than 50% of 'whole body' PON1 activity[13]. Animal studies carried out to-date provide convincing evidence that PON1 status plays a major role in determining sensitivity

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or resistance to OP compounds processed through the P450/PON1 pathway[14]. Initial evidence was provided by the observation that injection of an 'A-esterase concentrate' from rabbit serum into rats, could protect them from the acute toxicity of paraoxon given by i.v. injection[15]. Further correlative evidence came from studies that showed animals with low PON1 levels were more sensitive to specific OP compounds than animals with high enzyme levels. For example, birds, which have very low to undetectable PON1 activity, are more sensitive than various mammals to the acute toxicity of paraoxon, diazinon oxon and pirimiphos oxon[16,17]. Likewise, rabbits, which have a seven-fold higher serum PON1 activity than rats, are four-fold more resistant to the acute toxicity of paraoxon[18].

It is known that the acute toxicity of OPs is influenced by age, with substantial evidence indicating that young animals are more sensitive than adults[19,20]. The lower metabolic abilities of young animals appear to be a major determinant of their increased sensitivity to acute OP toxicity[20]. For example, in cattle, the toxicity of chlorpyrifos is 30-fold higher in calves than in adult animals, while for disulfoton, which is not metabolized by PON1, there was only a two-fold difference[21].

In the present study, we investigate the differential *in vitro* inhibitory effects upon PON1 activity of some drugs used in cattle during infectious diseases or inflammatory-like conditions.

Materials and methods

Sepharose-4B, L tyrosine, 9-aminophenanthrene, paraoxon, protein assay reagents and all chemicals for electrophoresis were obtained from Sigma Chem. Co. (Milan/Italy). All other general chemicals used were analytical grade and obtained from various sources.

Paraoxonase purification from serum

Bovine serum was isolated from fresh bovine blood collected in a dry tube. The blood samples were centrifuged at 1500 rpm for 15 min and the serum was collected. Different ammonium sulphate intervals, 0–100%, were determined for serum paraoxonase enzyme. Upon each stage, it was spinned at 15 000 rpm for 30 min and the total protein concentration and paraoxonase enzyme activity were determined in the each ammonium sulphate intervals. The precipitation intervals for paraoxonase enzyme were 60–80%. The precipitate was collected by centrifugation at 15000 rpm for 20 min, and redissolved in 100 mM Tris–HCl buffer (pH 8.0). The crude PON1 was then subjected to hydrophobic interaction chromatography. The PON1 solution was adjusted to 1M ammonium sulphate, and then loaded onto a hydrophobic gel column comprised of Sepharose-4B-L-tyrosine-9-aminophenanthrene which was synthesized as previously described by us for this enzyme purification[22]. The purification table was given in Table 2.

Paraoxonase enzyme assay

PON1 enzyme activity towards paraoxon as a substrate was quantified spectrophotometrically by the method described by Gan *et al.*[23]. The reaction was followed for 2 min at 37°C by monitoring the appearance of *p*-nitrophenol at 412 nm on a Biotek (Winooski, VT, United States) automated recording spectrophotometer. The final substrate concentration during enzyme assay was 2 mM, and all rates were determined in duplicate and corrected for the non-enzymatic hydrolysis. A molar extinction coefficient (ε) of 17,100 $M^{-1}cm^{-1}$ for *p*-nitrophenol at pH 8.0 in 100 mM Tris–base buffer was used for the calculation. One unit of PON1 activity is defined as 1 μmol of *p*-nitrophenol formed per minute under the above assay conditions.

In vitro inhibition studies

For the inhibition studies, PON1 activity was assayed by following the hydration of paraoxon in the presence of five different concentrations of various drugs. The drugs investigated were oxytocin, dexamethasone, atropine sulphate, gentamicin sulphate, sulfadoxine-trimethoprim, furosemid, metamizole sodium and toldimfos sodium. They were obtained from a local pharmacy in Balikesir / Turkey. Control PON1 activity was accepted as 100%, and the concentration of each drug causing 50% inhibition (IC_{50} values) of activity were calculated from inhibition curves (Figures 1–7 and Table 1).

Results and discussion

Oxidative stress affects PON1 activity, and there is an inverse relationship between lipid peroxidation and PON1[24]. Dairy cows are highly susceptible to oxidative stress which commonly occurs in late pregnancy and early lactation. During the transition period, increased production of reactive oxygen species is associated to processes of metabolic adaptation to a low-energy balance. An increased capability of milk production is associated with the changes of metabolic and energy homeostasis related to this, the blood concentration of PON1 can be relatively stable throughout the lifespan of an individual[25,26] though it can be influenced by disease state, diet, and other environmental factors. For example,

Table 1. IC_{50} values of drugs on PON1.

Drug name	IC_{50} (mg/mL)
Oxytocin	507.72
Dexamethasone	0.014
Atropine sulphate	0.041
Gentamicin sulphate	3.784
Sulfadoxin + trimethoprim	15.636
Furosemid	0.235
Metamizole sodium	26.777
Toldimfos sodium	11.08

PON1 levels fall after serious insults such as atherosclerosis, myocardial infarction[27,28].

Several factors affect the fate of drugs in animals[29]. Oxytocin, dexamethasone, atropine sulphate, gentamicin sulphate, sulfadoxin-trimethoprim, furosemid, metamizole sodium and toldimfos sodium drugs are all used in the treatment of cattle[30]. In our study the differential inhibitory effects of these drugs upon PON1 were determined *in vitro* for a better understanding of peroxidative damage and its relation to serum PON 1.

Oxytocin is a posterior pituitary hormone that acts directly on smooth muscle to produce rhythmic contractions and with this ability it is closely involved in lactation[30]. Because of this property, oxytocin is often injected into cattle that are under stress or unable to produce much milk to enhance milk production[31]. However, some research indicates that administering oxytocin to stimulate uterine contractions in pregnant cows can lead to increased early embryonic death[32]. The present results indicate that oxytocin does not affect the activity of serum PON1 as it showed a very high IC_{50} of 507.72 mg/mL (Table 1 and Figure 1).

Dexamethasone is a synthetic analogue of prednisolone, having similar but more potent anti-inflammatory therapeutic action and diversified hormonal and metabolic effects. Experimental animal studies have revealed it possesses greater anti-inflammatory activity than many steroids. It is used topically on the eye after cataract operations, on the skin for eczema and psoriasis, as well as intra-articularly for arthritis and osteoarthritis[33]. In this study, a strong inhibition of PON1 was observed (an IC_{50} of 0,014 mg/mL; Table 1 and Figure 2) with dexamethasone. PON1 plays an important physiological role in lipid metabolism, as it can hydrolyze oxidized lipids in the form of lipid hydroperoxides generated on lipoproteins such as HDL and LDL, thus providing protection against the development of oxidative stress. Usage of this drug could therefore result in lowered PON1 activity in cattle, which could potentially enhance production of reactive oxygen species (ROS), due to incomplete reduction of molecular oxygen to water as a consequence of electron leakage in electron transport chain.

A much more potent inhibition of PON1 was obtained with atropine sulphate with an IC_{50} of 0.041 mg/mL (Table 1, Figure 3). Atropine is a plant alkaloid that has been used extensively in most animal species as an anticholinergic agent, and atropine sulphate is used as an antidote in the treatment of organophosphate insecticide poisoning of cattle, horses and sheep, and in the treatment of nerve agent casualties requiring artificial

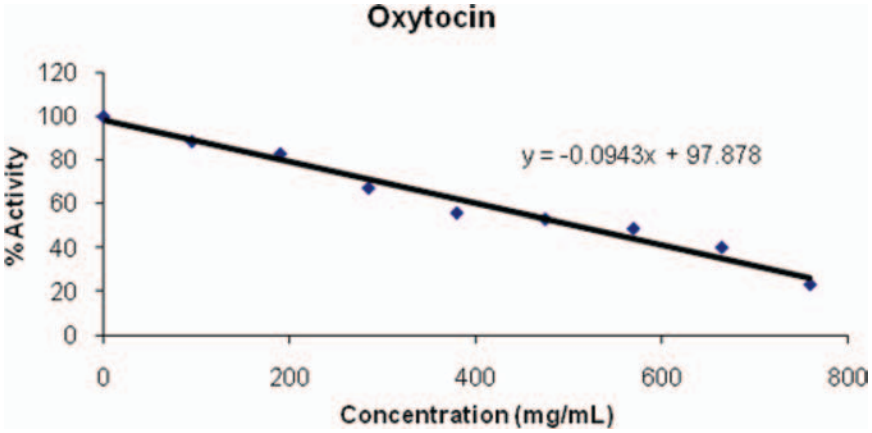

Figure 1. Inhibition of oxytocin on PON1.

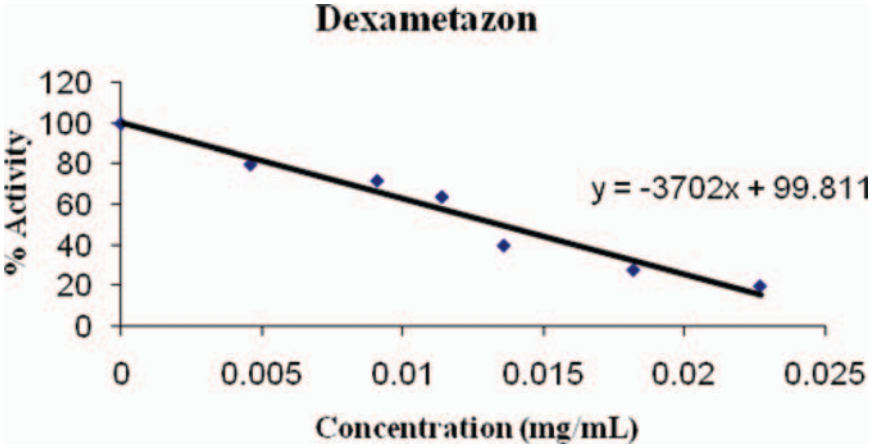

Figure 2. Inhibition of dexamethasone on PON1.

respiration[34]. As both atropine sulphate and PON1 are recognized to have a role in the metabolism of xenobiotics, through hydrolysis of the toxic oxon metabolites of organophosphorus compounds providing limited protection against chronic exposure to OP[35], this drug inhibition of PON1 is highly significant.

Gentamicin sulphate is an aminoglycoside antibiotic with a wide antibacterial spectrum that is commonly used in veterinary practice[36]. Although there is considerable information on the pharmacokinetics of this antibiotic in domestic animals[37], the Food and Drug Administration (FDA) recently issued warning letters to veterinarians for their illegal use of gentamicin sulfate[38]. The FDA has not approved gentamicin for use in cattle because of the propensity for these drugs to be retained in kidney tissue for long periods. But aminoglycoside nephrotoxicity is a significant problem that limits the clinical use of this important class of antibiotics. Insight into the mechanisms of aminoglycoside entry into tubular cells and subsequent cytotoxic events could provide important clues toward reducing the toxicity of these agents[39]. Thus it could be also related with paraoxonase activity because of this enzyme relation with xenebiotic metabolism as well. In our study we found an IC_{50} value of 3.784 mg/mL for PON1 inhibition by showed no better inhibition with comparing other drugs at the above (Figure 4, Table 1).

Sulfadoxine-trimethoprim is a potent antibacterial drug, and the reservoir of trimethoprim in the extravascular drug compartment, which is available for potentiation of the more slowly eliminated sulfadoxine, probably explains the high and prolonged antibacterial activity of the combination in ruminants[40]. In the literature, penicillin, oxytetracycline, and a trimethoprimsulfadoxine combination were compared as first choice antibiotics for the treatment of acute bovine respiratory disease in weaned beef calves[41]. The selection of an appropriate antibiotic and the evaluation of its success in the treatment of respiratory disease is an important consideration. Initial field trials with a potentiated sulfonamide suggested a high level of efficacy in the treatment of bacterial diseases of cattle and pigs[42]. In our study there was no significant inhibitory effect of this drug on PON1 activity (IC_{50} of 15.636 mg/mL; Table 1, Figure 5).

Furosemid is a loop diuretic used in the treatment of congestive heart failure and edema. In cases of edema involving cardiac insufficiency, the continued use of heart stimulants such as digitalis or its glycosides is

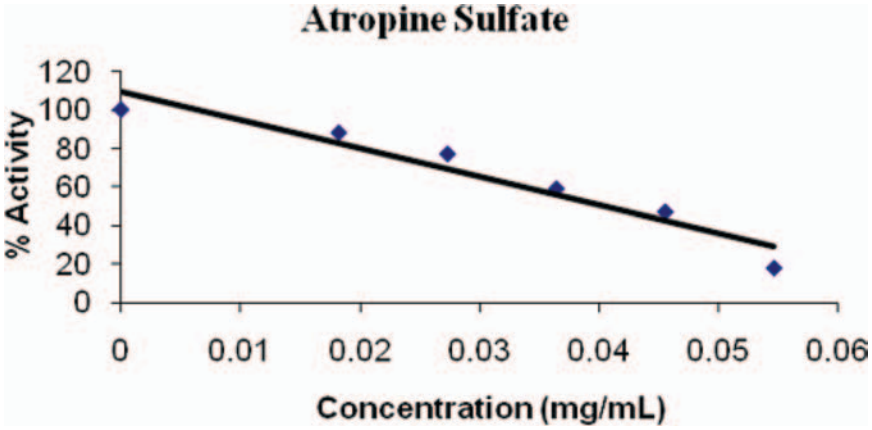

Figure 3. Inhibition of atropine sulphate on PON1.

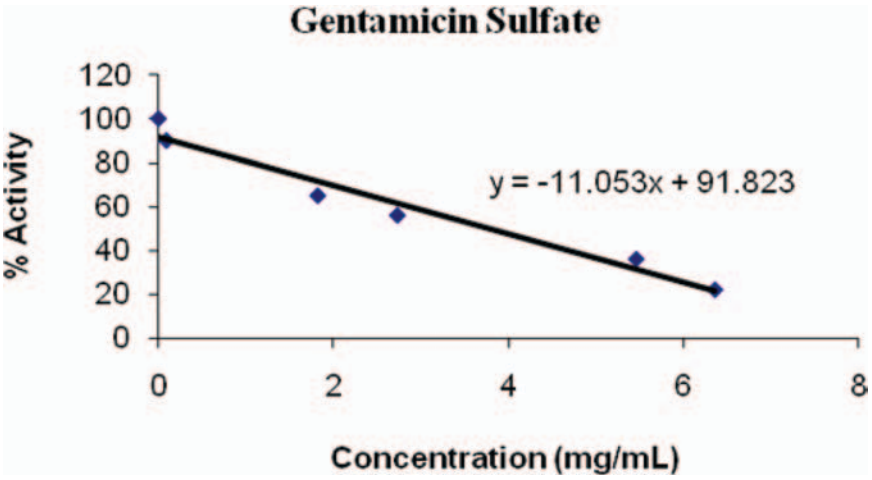

Figure 4. Inhibition of gentamicin sulphate on PON1.

indicated[43]. It has also been used to prevent thoroughbred and standard bred race horses from bleeding through the nose during races. As with many diuretics, it can cause dehydration and electrolyte imbalance, including loss of potassium, calcium, sodium, and magnesium[44]. Furosemid had an effective inhibition of PON1 with an IC_{50} of 0.235 mg/mL (Figure 6, Table 1).

Metamizole sodium as a non-steroidal, anti-inflammatory drug commonly used in many countries as a powerful painkiller, for pain originating from spasms of smooth muscles and fever reducer[45]. Metamizole preparations are indicated for use in horses, cattle and swine as an adjunct to therapy in many inflammatory conditions of musculoskeletal and locomotor systems. It is better known under the names Dipyrone, Analgin, Novalgin, and Melubrin. Here in this study, this drug gave a poor inhibition of PON1 with an IC_{50} of 26.777 mg/mL (Table 1 and Figure 7).

Toldimfos sodium is the sodium salt of 4-dimethyl-amino-2-methyl-phenyl-phosphinous acid, a derivative of phosphoric acid. It is indicated for the treatment and prophylaxis of diseases which arise in connection with parturition and the peri-partum period, developmental and nutritional disorders in young animals, disorders of bone growth and tetasy or paresis caused by disorders of calcium, magnesium and phosphorous metabolism[46]. Here we found only a poor inhibition of serum PON1 (an IC_{50} value of 11.08 mg/mL), compared to the effects of dexamethasone, atropine sulphate and furosemid (Table 1 and Figure 8).

In the literature there are also some studies about PON1 purification and its interaction with sulfonamides, Supuran and his collegues had 302-fold purification with a final specific activity of 4775 U/mg and a yield of 32% with using six sulfonamides dose-dependently decreased the activity of hPON1 with inhibition constants in the millimolar-micromolar range by using several purification steps[47]. But in this present study we had 1690 fold purification and a 14922.8 U/mg specific activity by using a hydrophobic gel column comprised of Sepharose-4B-L-tyrosine-9-aminophenanthrene in a single step. Also we determined the inhibition effects of some cattle drugs on PON1 level. Another study had showed evaluation *in vitro* and *in vivo* effects of the intravenous anesthetics, etomidate, propofol, and ketamine, on the activity of human serum paraoxonase (hPON1[48]). and hPON1 was purified in three steps with a high specific activity, and anesthetics inhibited PON1 activity.

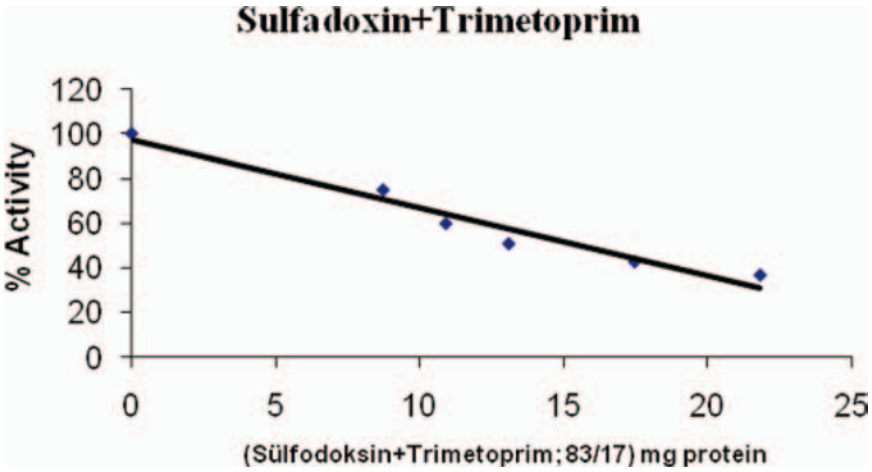

Figure 5. Inhibition of sulfadoxin-trimethoprim on PON1.

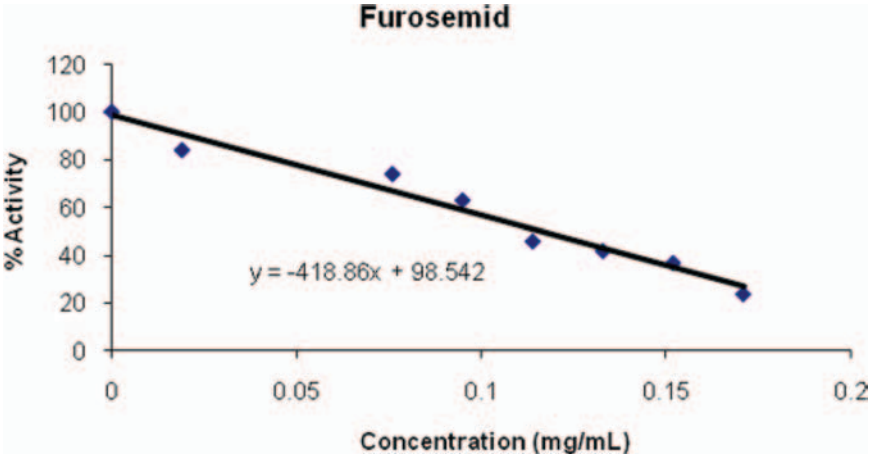

Figure 6. Inhibition of furosemid on PON1.

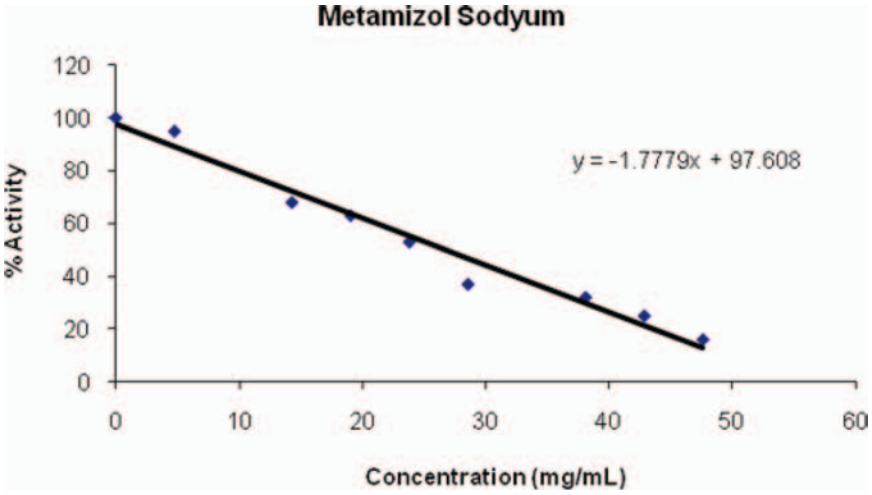

Figure 7. Inhibition of metamizole sodium on PON1.

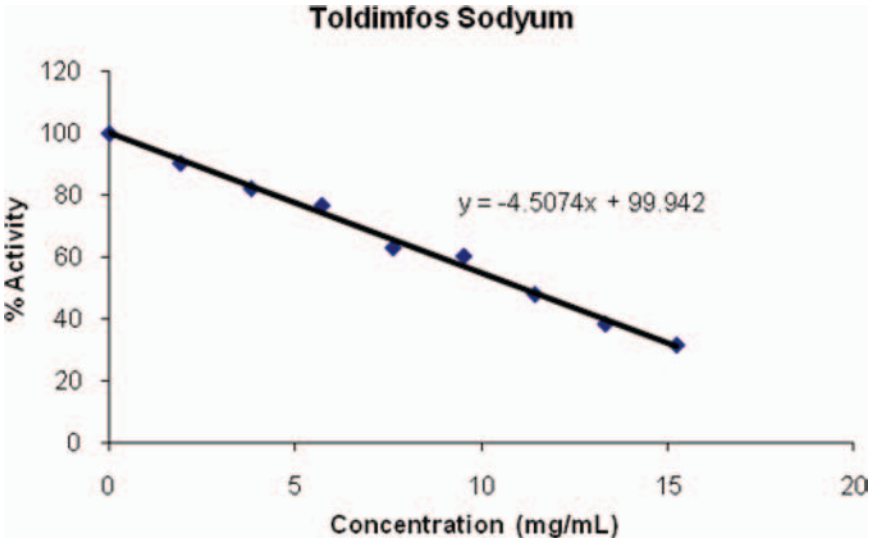

Figure 8. Inhibition of toldimfos sodium on PON1.

Comparing their results of our study, the purification fold of PON 1 is higher than their method. Beydemir *et al.* also had another different study with PON 1 purification using very simple methods and investigation of the interactions between the enzyme and some commonly used antibiotics[49]. The drugs were determined to decrease the enzyme activity at quite different concentrations. We also used gentamicine sulfate for having a comparison on PON1 level that is why recently this kind of studies have more attractions for a better understanding of drug metabolism. According to our findings, most potent and significant inhibition was displayed by dexamethasone, atropine sulphate and furosemide. But in their study some of the antibiotics exhibited different inhibitory effects on hPON1.

Conclusions

PON1 has been reported to be involved in drug metabolism and is used for drug inactivation[50]. In this study, PON1 is purified by an hydrophobic gel column comprised of Sepharose-4B-L-tyrosine-9-aminophenanthren (Table 2). The efficacy of drug inhibition of PON1 activity was very variable across the panel of drugs tested with IC_{50} values varying between 0.014–507.72 mg/mL. When listed in decreasing order of inhibitory potential the drugs are: dexamethasone (0.014 mg/mL), atropine sulphate (0.041 mg/mL), furosemid (0.235 mg/mL), gentamicin sulphate (3.784 mg/mL), toldimfos sodium (11.08 mg/mL), sulfadoxine-trimetoprim (15.636 mg/mL), metamizol sodium (26.777 mg/mL) and oxytocin (507.72 mg/mL) (Table 1). Within these results, it is clear that significant inhibition was observed with dexamethasone, atropine sulphate and furosemid.

Turk *et al.*[51] demonstrated that serum PON1 activity is reduced in early postpartum dairy cows, and oxidative stress is considered to contribute to various disorders in this period thus long periods with lower concentrations of PON1 activity, which defends the animal against oxidative stress, may increase the risk of

Table 2. Summary of the purification of bovine serum paraoxonase.

Step	Volume (ml)	Activity (U/ml)	Total activity (U/ml)	Protein amount (mg/ml)	Total protein (mg)	Specific activity (U/mg)	Overall yield %	Overall purification (Fold)
Serum	33	61.4	2026.2	8.641	285.2	7.11	100	–
Ammonium sulfate fractionation	14	83.51	1169.14	9.458	132.4	8.83	57.7	1.24
Hydrophobic interaction chromatography	2	117.89	235.78	0.0079	0.0158	14922.8	11.6	1690

oxidative damage, particularly at the lipoprotein level. Beydemir *et al*.[52] reported that dexamethasone inhibited *in vitro* human PON1 activity. They found that IC50 value was 1.106 mM for dexamethasone. When this study was compared to our results, both the results were given similar inhibition values. The results showed that dexamethasone is a potent inhibitor. So, the drug must be used carefully and the dosage closely monitored to decrease side effects. In addition, Sinan *et al*.[53] performed some experiments about the *in vitro* effects of sodium ampicillin, ciprofloxacin, and clindamycin phosphate on PON1 purified from human serum. The study found that sodium ampicillin, ciprofloxacin, and clindamycin phosphate were potent inhibitors for human serum PON1, and IC_{50} values were 27.51 mM, 0.313 mM and 0.902 mM, respectively. Having a comparison to our values, it is seen these drugs had more inhibition effects than our results.

The objective of this study was to determine the relationship between serum PON1 activity level and cattle drugs for a better understanding of peroxidative damage in relation to serum PON1 activity and how various drug treatments might compromise these processes which may cause some side effects on health problems, inflammatory conditions, plasma metabolic measures, and milk yield on bovines. The ability of some cattle drugs to significantly inhibit PON1 activity *in vitro* indicate that further studies, especially *in vivo*, are needed to better understand the potential interrelationships between drug treatments, PON1 activity, liver function and oxidative stress, and subsequent effects on animal health.

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Declaration of interest

The authors report no conflict of interest.

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