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Pharmacokinetic studies of α-difluoromethylornithine in rabbits using an enzyme-linked immunosorbent assay

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Abstract: Using an inhibition immunoassay, the pharmacokinetics of DFMO have been evaluated. After intravenous infusion, DFMO concentration in serum is observed to reach a maximum after 2–3 h followed by a decrease. This profile is consistent with the formation of a covalent adduct between DFMO and the enzyme which it irreversibly inhibits. The adduct was isolated by immunoaffinity chromatography.

Keywords: *Alpha-difluoromethylornithine; immunoassay; immunoaffinity chromatography; pharmacokinetics; irreversible enzyme inhibitor.*

Introduction

Alpha-difluoromethylornithine (DFMO) (Ornidyl (eflornithine hydrochloride), Marion Merrell–Dow, Kansas City, MO, USA) is a specific irreversible inhibitor of the enzyme ornithine decarboxylase (ODC) which is the initial rate-controlling enzyme in the polyamine synthetic pathway [1]. Ornithine, the natural substrate of ODC is catalytically decarbonylated by ODC to produce carbon dioxide and the diamine, putrescine. Putrescine further metabolizes to the polyamines, spermidine and spermine. DFMO is enzymatically decarboxylated and covalently bound by ODC and the inhibition is, therefore, irreversible [2].

Intracellular polyamine levels are highly dependent on the activity of ODC. The ODC levels are normally low in most tissues. The level of the enzyme as well as the levels of the polyamines are observed to increase upon the initiation of cell proliferation [3, 4]. Experimental evidence has suggested that increased ODC activity provides one of the most reliable methods for detecting the transformation of cells into a tumour [5].

DFMO is currently being investigated as a potential anti-neoplastic agent because of its ability to irreversibly inhibit ODC. Single agent anti-tumour activity of DFMO has been reported both *in vitro* and *in vivo* [6]. DFMO has been shown to inhibit the induction of cancer in various animal models when given orally (7, 8). In addition, DFMO is known to enhance the anti-tumour activity of other anti-cancer drugs such as tamoxifen and vindesine in combined treatment [9, 10]. Although this drug has been investigated as a cancer chemotherapeutic and cancer prevention agent [11, 12] it has been approved by the Food and Drug Administration for the treatment of the meningoencephalic stage of the African sleeping sickness, a tropical disease caused by *Trypanosoma brunei gambienze* [13, 14].

The utilization of DFMO as an anti-cancer drug requires an assay for DFMO in biological samples to conduct pharmacological studies in human or animal subjects [15]. The two methods currently available for the assay involve HPLC or ion chromatography with post-column derivatization with *o*-phthalaldehyde [15, 16]. Recently, another HPLC method with pre-column fluorogenic derivatization with naphthalene-2,3-dicarboxyaldehyde/cyanide (NDA/CN^-) has been reported (Osei, A. *et al.*, unpublished results). All of these methods require sophisticated and expensive

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instrumentation and lengthy sample preparations. Therefore, development of a simple, sensitive assay method for DFMO is highly desirable. The best suited assay method for this purpose would be an enzyme-linked immunosorbent assay (ELISA).

Although ELISA methods are generally simpler and faster than the chromatographic methods, and are particularly suitable for biological samples, their application to assay small drug molecules has been very limited. This is possibly due to the difficulties associated with the preparation of the antibodies to the particular drug. Small drug molecules such as DFMO are not immunogenic and do not elicit a good immune response. They are also excreted from the body rapidly making them less available for the immune system.

The ELISA developed here offers several advantages over the existing methods for DFMO. It is highly sensitive and requires no sample preparation except a dilution of the samples. The matrix of the sample does not affect the assay as in the chromatographic methods involving the derivatizing agents which indiscriminately derivatize the amino groups of the proteins present in plasma and thereby necessitate the use of complex chromatographic separation techniques.

Experimental

Chemicals and reagents

Bovine serum albumin (BSA), keyhole limpet hemocyanin (KLH), 1-ethyl-3(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) DL-ornithine hydrochloride, putrescine dihydrochloride and glutaraldehyde were purchased from the Sigma Chemical Co. (St Louis, MO, USA). CDI-activated Reactigel (HW-65F) was obtained from Pierce Chemical Co. (Rockford, IL, USA). ELISA grade horse radish peroxidase (HRP) was purchased from Biozyme Laboratories (San Diego, CA, USA). HRP conjugated goat anti-rabbit and goat anti-rabbit Fc specific antibodies were purchased from Organon Teknika–Cappel Corp (Durham, NC, USA). The substrate for HRP, tetramethyl benzidine (TMB) was purchased from Kirkegaard and Perry Inc. (Gaithersburg, MD, USA). DFMO and the serum DFMO samples obtained from cancer patients participating in a Phase I clinical trial, were a generous donation from Milan Slavik, M.D. of the VA Medical Center (Wichita, KS, USA). All other reagents were of analytical grade. Solutions were prepared in water obtained from a Barnstead Nanopure II system.

Instruments

ELISAs were carried out in 96-well plates (Corning, NY, USA) and the absorbance was read with a V_{max} microplate reader (Molecular Devices, CA, USA) at dual wave length 450–650 nm. A Shimadzu UV/VIS-160 spectrophotometer was used for spectroscopic measurements. Affinity purifications were carried out in homemade columns using a Rainin Rabbit peristaltic pump (Rainin Instruments, MA, USA) and a ISCO flow path monitor at 280 nm (ISCO Instruments, NE, USA).

Preparation of hapten-protein conjugates

DFMO is essentially an amino acid with molecular weight of 236.65 Da. Therefore, it was necessary to conjugate DFMO to a large carrier protein such as KLH, which has a molecular weight of about 3×10^6 Da for immunization. The coupling was done by two methods, one through the -COOH group of DFMO and the other through the α-NH_2 group. The coupling through the -COOH group was achieved by enhanced carbodiimide coupling [17] as described elsewhere [18]. Briefly the procedure is as follows: EDC (100 µl, 0.1 M in the final volume) in 0.1 M phosphate buffer (PB), pH 7.4 was added to a mixture containing DFMO (3 mM), sulfo-NHS (5 mM) and KLH at a 3 mg ml^{-1} concentration in PB (pH 7.4) in a total volume of 12.4 ml. After allowing to react overnight with stirring at room temperature, the reaction mixture was extensively dialysed against 0.01 M phosphate-buffered saline (PBS) to remove excess reagents. The conjugate thus prepared (KDE) was stored frozen in aliquots until use.

Coupling through the amino group was done by the method described by Zegers *et al.* [19] using glutaraldehyde to link DFMO to KLH. Briefly, in this method KLH was dissolved in PBS and dialysed against 0.2% glutaraldehyde (GA) at 4°C for 16 h. The GA activated KLH was then dialysed against several volume changes of 0.01 M PBS to remove the excess GA. DFMO was dissolved in water and added to this dialysed KLH and allowed to react for 16 h at 4°C. The mixture was then reacted with 100 µl of 0.1 M lysine to block the active sites

on the carrier. The conjugate (KDG) was then dialysed against 0.01 M PBS for 48 h.

DFMO was conjugated to bovine serum albumin (BSA) for screening purposes so that antibodies against the carrier used for immunization, KLH, can be screened out. BSA-DFMO conjugates were prepared by the enhanced carbodiimide coupling method (labelled as BDE) to screen the antisera from the KDG group and by the glutaraldehyde method (BDG) to screen the antisera from the KDE group. This way, the antibodies made against the carrier and the link between the carrier and DFMO can be screened out. In addition, an ornithine-BSA conjugate was prepared by the glutaraldehyde method for the affinity purification of the antibodies.

Production of polyclonal antibodies

Polyclonal antibodies were made in rabbits according to a standard protocol [20]. A total of four female New Zealand White rabbits, two with each conjugate (KDE and KDG) were initially immunized intradermally at five sites, each site with 100 μl of the conjugate in Freund's Complete Adjuvant (FCA) (1:1). At two week intervals, three booster immunizations with 200 μl of the conjugates in Freund's Incomplete Adjuvant (FIA) or in saline were given intramuscularly. Two weeks after the last immunization, the rabbits were bled from the ear vein and the antibody titer was determined.

Screening of antibodies

The antisera were screened by an ELISA. Microtiter plates were coated with BDE or BDG at 10–30 μg ml^{-1} (100 μl/well) in 0.1 M carbonate buffer (pH 9.6). Plates were covered and incubated at 37°C for 2 h. After washing with 0.01 M phosphate buffered saline (pH 7.4) containing 0.05% Tween-20 (PBST), 100 μl of 20-fold diluted antiserum was added to each well and incubated for 1 h at 37°C. After washing the plates with PBST, 100 μl of peroxidase conjugated goat anti-rabbit (gtxrb-HRP) diluted 1:2500 with PBST containing 0.2% BSA was added to each well and incubated 1 h at 37°C. Finally after another washing step, 100 μl of the TMB substrate was added. After 20 min the enzyme reaction was quenched by adding 50 μl of 0.1 M HCl and the absorbance at two wavelengths (450–650 nm) was measured.

Purification of antibodies. Purification of the antibodies was carried out in three steps. First the immunoglobulin (Ig) fraction containing the crude antibody was precipitated from the antiserum by ammonium sulphate precipitation. The IgG fraction was obtained from the Ig fraction by anion exchange chromatography using DEAE-Sephacel with 0–300 mM NaCl linear gradient in 10 mM Tris buffer (pH 8.0). The IgG fraction was then collected, concentrated and dialysed against 0.1 M PB (pH 7.4). This IgG fraction was next purified by affinity purification using an affinity column of BDG to obtain the antibodies recognizing the DFMO structure. Since DFMO, ornithine and putrescine share the same ϵ-NH_2 side chain, these antibodies may cross-react with the two latter species. In order to separate the antibodies which are specific only to DFMO, the antibody solution thus purified was then applied to a second affinity column containing BOG coupled support. Only antibodies that are specific to the moiety containing the difluoromethyl group of DFMO will then be eluted and antibodies crossreacting with ornithine will be retained by the column. The complete experimental procedure is described elsewhere [13]. The concentration of the DFMO-specific antibodies was determined by the absorbance at 280 nm. The purified antibody was always stored in PBS.

Characterization of the antibodies. The affinity purified antibody was then screened as described before against BSA and BOG to determine the cross reactivity to BSA and ornithine. The apparent affinity constant was determined from the same ELISA by serially diluting the antibody [21]. The affinity of the antibody for DFMO in solution was studied by an inhibition ELISA. This ELISA was carried out by incubating different concentrations of the affinity purified antibody (6.25×10^{-7}–3.75×10^{-11} M) with varying concentrations of DFMO (from 0.02 μM to 2.0 mM) in solution. After a 2 h incubation period at 37°C, the samples were applied to a BDG-coated microtiter plate and the normal ELISA procedure performed.

Competitive and inhibition immunoassay procedures for sample analysis

In the competitive assay procedure, the DFMO specific antibodies were adsorbed on the plate through a capture antibody in order

to capture the antibody in the proper orientation. Microtiter plates were first coated with 100 μl of the capture antibody, gtxrb-Fc specific antibodies (10 μg ml^{-1}). The purified DFMO-specific antibody (100 μl of 4 μg ml^{-1}) was then added to the wells. Standards or samples (50 μl) were mixed with 50 μl of the DFMO-HRP conjugate (HDG) and added to the antibody coated wells. After a 1 h incubation period at 37°C the TMB substrate was added and the absorbance was measured as described before. Concentrations of the antibody and HDG were optimized to obtain a wider linear range in the assay.

In the inhibition method the plates were first coated with BDG. The standards or samples (50 μl) were mixed with 50 μl of 4 μg ml^{-1} of purified antibody and incubated at 37°C for 1 h. The incubated samples were then added to the BDG-coated microtiter plate and incubated for 1 h at 37°C. The plates were washed and 100 μl of 1:2500 diluted gtxrb-HRP was added. After another 1 h incubation at 37°C, TMB substrate was added and the absorbance was measured. Concentrations of BDG, antibody and the gtxrb-HRP were optimized to obtain a wide linear range. The calibration curves were obtained by analysing the data by a four-parameter logit curve fitting procewdure which also calculated the concentrations of the unknowns and the controls.

The human serum samples containing DFMO and the rabbit serum samples from the pharmacokinetic studies were assayed by the inhibition method. These samples were diluted in pooled normal human plasma and the antibody solution (4 μg ml^{-1}) was also prepared in normal human plasma. The gtxrb-HRP was diluted (1:2500) in PBST containing 0.2% BSA. Control samples were prepared by adding known amounts of a stock DFMO solution (200 μM) in normal human plasma.

Pharmacokinetic studies in rabbits

Three New Zealand rabbits were administered intravenously a 10 mg kg^{-1} body weight dose of DFMO in sterile saline intravenously in one ear. The rabbits were bled from the other ear by venapuncture at time intervals of 0, 0.25, 0.5, 0.75, 1, 2, 3, 4, 6, 8, 10, 12 and 24 h. All serum samples were prepared soon after blood collection and stored frozen at −40°C until assayed.

Identification of the metabolite

Two rabbits (used in the pharmacokinetic experiment) were given at 50 mg kg^{-1} body weight dose of DFMO as in the pharmacokinetic studies. Three hours later the rabbits were bled from the other ear. Blood from both rabbits were pooled and serum was separated. This isolated serum was then passed through an affinity column with immobilized DFMO-specific antibody at a very low flow rate to allow the complete capture of the metabolite by the antibody. The bound fraction was then eluted with a 1:1 mixture of methanol–water. The eluate was found to be protein-bound. This protein-bound fraction was then evaporated to dryness on a rotary evaporator. Protein from this metabolite sample was precipitated either with 20% formic acid or 50% methane sulphonic acid and the supernatant was concentrated and subjected to mass spectroscopic identification.

Results and Discussion

The antisera obtained by immunizing the rabbits with KDE were screened against DFMO conjugated to BSA through the amine group and the antisera produced against KDG were screened against DFMO conjugated to BSA through the -COOH group. This screening protocol eliminates the antibodies specific to the carrier and to the link. The antisera produced against KDE had a very high titer (~40,000) against the BSA-DFMO conjugate whereas the titer was low for the antisera against KDG (~600). Antibodies purified from the antisera produced against KDE were, therefore, used for further studies.

The antibodies purified by ion-exchange did not show any cross reactivity toward BSA. The $(-CH_2)_3-NH_2$ part of the DFMO molecule is similar to the $\epsilon-NH_2$ side chain of lysine and since, BSA contains lysine residues, it can be concluded that the antibodies are not specific to the epitope containing the $\epsilon-NH_2$ side chain. Selective affinity purification of the antibodies by first passing through an affinity column of BDG and then through another column of BOG eliminated the cross reactivity to ornithine to a large extent (Fig. 1). The affinity purified antibody had minimal cross reactivity to ornithine (2–4%) indicating that the antibody involves mainly the epitope containing the difluoromethyl group of the molecule. The apparent affinity constant for DFMO was

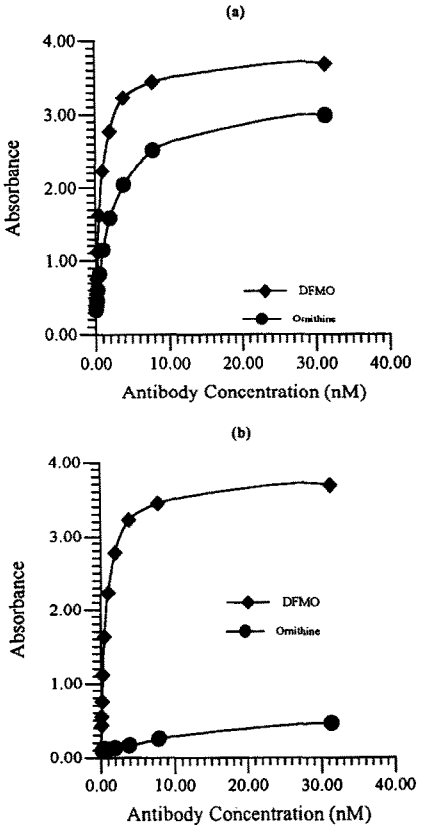

Figure 1
Cross reactivity of antibody to ornithine (a) before and (b) after affinity purification. Serially diluted antibody solutions (100 μl of 3.125×10^{-8}–1.5×10^{-11} M) were incubated in BOG and BDG (10 μg ml^{-1}) coated microtiter plates at 37°C for 1 h and then the normal ELISA procedure was followed.

found to be 2.0×10^9 M^{-1} [18]. The inhibition ELISA showed that DFMO in solution binds to the antibody with equal affinity.

The assay was carried out in two formats. In the competitive format (Fig. 2a), analyte in the sample competes with the enzyme-labelled analyte for the antibody on the microtiter plate. The amount of labelled analyte bound to the plate is then determined. A competitive assay is the commonly used assay format for small haptens such as DFMO.

In the inhibition format (Fig. 2b) the analyte in the sample is first allowed to bind to an excess of antibody in the solution phase and the amount of free antibody is detected on a microtiter plate. Both formats are indirect measurements of the analyte content in the sample.

All the standards and control samples were prepared in pooled human plasma to avoid any matrix effects. Both competitive and the inhibition formats had a linear range of 1.0–100 nmol ml^{-1} and a detection limit of 0.5 nmol ml^{-1}, but the precision of the competitive assay was very poor. On the other hand, the inhibition assay provided high precision. A typical CV of an ELISA is about 10–20%. The inhibition assay format used here always had a CV of <5%. Therefore, all the samples were assayed by the inhibition method. The precision and accuracy data for the inhibition assay are given in Table 1.

The assay showed no interference from other structurally similar endogenous amines, namely ornithine and putrescine. Cross reactivity which is the main interference has been eliminated by selective affinity purification of the antibodies. The affinity purified antibodies showed minimal cross reactivity to ornithine (~2–4%) and no reactivity to putrescine. The response curves for DFMO, ornithine and putrescine are shown in Fig. 3.

The serum samples obtained from patients who participated in a phase I clinical trial were assayed by the inhibition ELISA. These plasma samples have previously been assayed by a commercial amino acid analyser (derivatization with orthophthaldehyde, LOD–1 μM) and by a chromatographic method (derivatization with naphthalenedialdehyde–CN^-, Osei, A. *et al.*, unpublished results). Samples were used as they are without any sample preparation. It was necessary to dilute some samples with pooled human plasma to fall in the linear range of the assay. The values obtained by the ELISA method were in good agreement with the values obtained by both chromatographic methods (Fig. 4) which shows that the ELISA could be used as a valid method to study the pharmacokinetics of DFMO.

Pharmacokinetics were studied by injecting DFMO into rabbits ($n = 3$) intravenously. Blood samples were drawn at measured intervals and the serum was separated and assayed by the inhibition assay as described before. The pharmacokinetic curve obtained by plotting the concentration of DFMO vs time (Fig. 5) showed a rapid increase of DFMO in plasma initially and a maximum around 2–3 h after the infusion of DFMO. This behaviour suggests that the drug is initially bound to the enzyme, ODC making it inaccessible to the anti-DFMO antibody. The DFMO is released later in a modified form which can react with the antibody.

Separation of these species responding to the

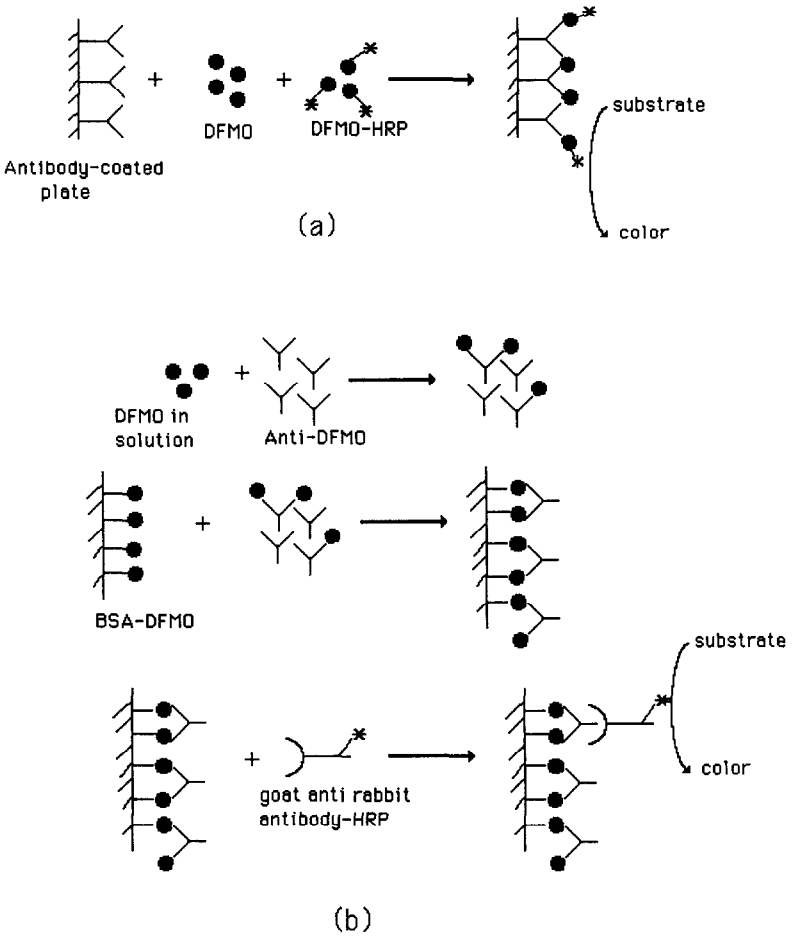

Figure 2
The assay schemes for DFMO (a) competitive ELISA format (b) Inhibition ELISA format.

Table 1
Precision and accuracy data for the assay

	DFMO Concentration (nmol ml^{-1})		
	10.0	40.0	80.0
Within run (n = 3)			
Mean	10.3	40.9	80.7
SD	0.2	0.7	0.9
CV%	1.9	1.7	1.2
Between run (n = 5)			
Mean	10.9	41.4	82.1.
SD	0.4	1.6	3.4
CV%	3.9	3.8	4.1

antibody was accomplished by passing the pooled sera through an affinity column of immobilized antibody as described above. The eluate was found to be protein-bound.

Mass spectroscopic analysis of this sample after the precipitation of protein revealed that these species have a considerably higher molecular weight than the parent compound. Tandem mass spectra gave a cluster of peaks

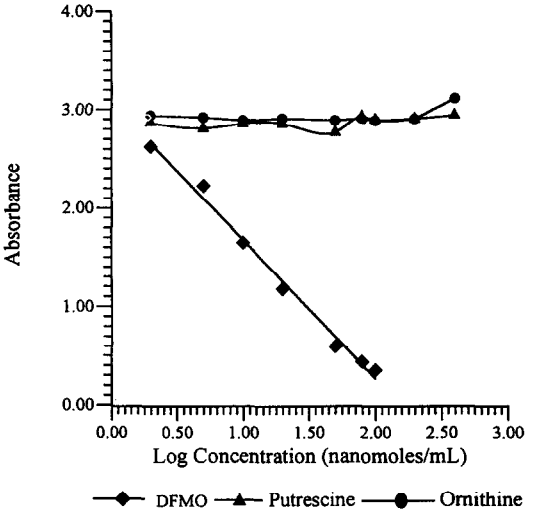

Figure 3
Response curves for DFMO, ornithine and putrescine. Samples containing 0–400 nmole ml^{-1} of each analyte were incubated with 50 µl of 4 µg ml^{-1} of antibody for 1 h at 37°C; Each data point is a mean of five measurements.

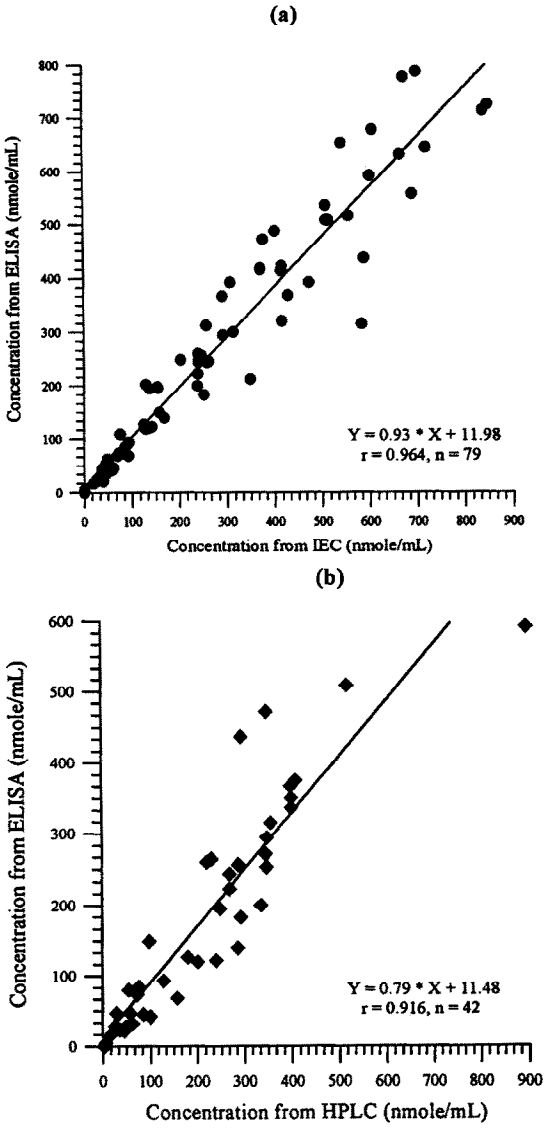

Figure 4
Correlation of serum DFMO concentrations by ELISA and (a) Commercial amino acid analyser (IEC) and (b) HPLC methods.

with a mass around 750–800 molecular weight suggesting the possible association of the metabolite with a peptide of about five–six amino acid residues.

Negative ion chemical ionization of a thermally evaporated dip probe compared the metabolite and the parent drug for fluoride. Fluoride profiles confirmed that the metabolite is an organic molecule containing fluoride. This implies that either these species are mainly the parent drug associated with a peptide or a metabolite containing the difluoromethyl group.

The techniques used for the separation and identification of the metabolite were too gentle to produce peptide fragments from a protein-bound parent drug. Hence, we can conclude that this peptide containing five–six amino acid residues was generated by the metabolism of the parent drug.

The irreversible inhibition of ODC by DFMO occurs first, by the decarboxylation of DFMO by ODC to generate an intermediate carbanionic species which induces the elimination of the fluoride group to form a reactive imine [2]. This reactive imine then alkylates a nucleophilic residue at or near the active site of the enzyme to covalently bind DFMO to the enzyme. ODC is a relatively short-lived enzyme and has a half-life of about 15–30 min [1]. Short-lived enzymes such as ODC are very sensitive to proteolytic attack [22]. It may be possible that the metabolite is a small peptide fragment of ODC containing irreversibly bound DFMO or DFMO being cleared to the serum. Further studies are needed to identify the exact structure of this metabolite and such studies are underway.

Conclusion

The immunoassay described here offers several advantages over the chromatographic methods available for this analyte. Chromatographic methods require extensive sample preparation and derivatization of the samples. Since the derivatizing agents used in the chromatographic analysis are not selective, complex separation techniques are required. This ELISA does not need any sample preparation other than a dilution step. The accuracy and the precision of the method is comparable to the values reported in literature for the chromatographic methods. ELISA methodology has been used successfully for the rapid and sensitive assay of a dopamine agonist at the 8 pg ml^{-1} level [23].

Since DFMO is essentially an amino acid derivative, producing monoclonal antibodies to such a molecule is very difficult and time consuming. We have been able to eliminate the cross reactivity of the polyclonal antibodies produced against DFMO by selective fractionation of the antibody population using immunoaffinity columns. Since it has been shown that the antibodies to DFMO show no cross reactivity to similar amines, this ELISA is free from the interferences from these amines.

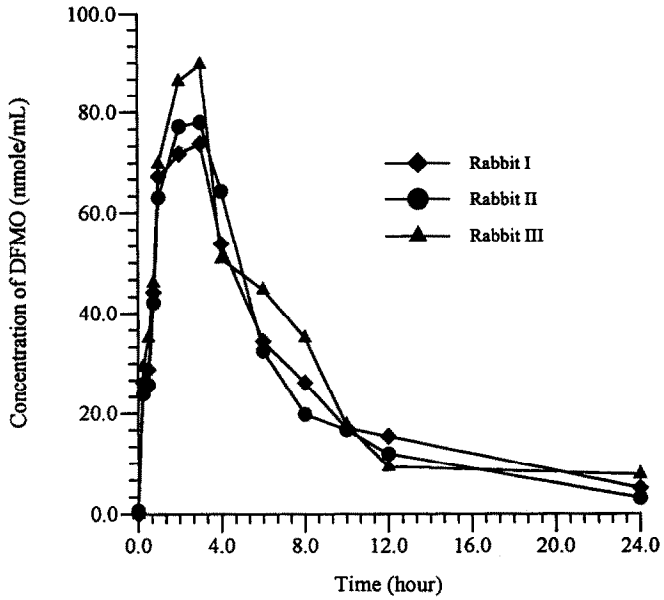

Figure 5
Pharmacokinetics of DFMO in rabbits. Three rabbits, each with a dose of 10 mg kg^{-1} of body weight were infused intravenously.

The affinity of the antibody to the -CHF_2 group has been used to our advantage to capture and separate the metabolite of DFMO.

In conclusion, we have developed a rapid, simple highly sensitive enzyme immunoassay method to assay DFMO in plasma. The assay has a very high precision compared to normal ELISA assays. Also, this assay was capable of revealing a metabolite or a protein-bound drug which was not seen by chromatographic methods.

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