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Modification of allergenicity and immunogenicity of formate dehydrogenase by conjugation with linear mono methoxy poly ethylene glycol: Improvement in detoxification of formate in methanol poisoning

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Abstract

Background: Single bolus intravenous infusion of native formate dehydrogenase (FD), isolated from *Candida boidinii* was found to eliminate formate, a highly toxic metabolite in methanol poisoning. In order to prevent immunological reactions which might be produced by multiple dosing of formate dehydrogenase and to prolong the serum half life of the enzyme, the *N*-hydroxysuccinimidyl ester of methoxy polyethylene glycol propionic acid (mPEG-SPA 5000) was conjugated to native formate dehydrogenase.
Method: PEGylation reactions were run at 20 °C for 30 min in a reaction buffer (0.2 mol/l sodium phosphate buffer, pH 8.3). The PEGylated molecules were purified from unreacted PEG with Amicon Ultra-4 (10 K) and by Sephacryl S-300 HR gel-filtration chromatography. Unreacted formate dehydrogenase molecules were removed by DEAE Sepharose FF anion-exchange chromatography. PEG–FD enzyme molecules obtained from reacting ratio of FD/PEG of 1/40 had an enzyme activity of 68% of unmodified enzyme. Immunogenicity of PEGylated and native enzyme was evaluated by ELISA. Allergenicity was evaluated by active systemic anaphylaxis and passive cutaneous anaphylaxis tests. In vivo efficacy of PEG–FD or native FD was comparatively evaluated by single intravenous administration of PEG–FD or native FD in folate deficient methanol intoxicated albino rats along with Carbicarb buffer infusion. Methanol and formate were estimated at specific time points respectively with HPLC and fluorescence spectrophotometer.
Result: PEG–FD had comparatively longer half life and lower immunogenicity than native FD. PEG–FD had better in vivo efficacy than native FD in eliminating the formate.
Conclusion: Conjugation of mPEG-SPA 5000 with native FD reduces its immunogenicity and increases its efficacy in detoxification of formate in methanol poisoning.

Keywords: PEGylation; Formate dehydrogenase; Formate; Methanol; Immunogenicity

1. Introduction

Methanol is well absorbed from the gastrointestinal tract mucosa as well as through the skin and lungs. Apart from oral ingestion, both inhalation and transdermal exposure can result in toxicity. It should be noted that the highest morbidity and mortality have been associated with deliberate or accidental oral ingestion of methanol-containing mixtures [1]. It is one of the major adulterants of illicit liquor in India causing blindness and death [2]. In mammalian species, methanol is metabolized to formaldehyde in the liver and by subsequent oxidative steps, formic acid and carbon dioxide are formed [3,4]. The metabolism of formate is mediated through folate dependent pathway [5]. Humans (and non-human primates) are uniquely sensitive to methanol poisoning because of their low liver folate content [6]. Specifically, formic acid is the toxic metabolite responsible for the metabolic acidosis observed in methanol poisoning in humans and in folate-depleted rodents [4]. Formic acid is believed to be

Abbreviations: FD, formate dehydrogenase; PEG FD, pegylated formate dehydrogenase; i.p., intra peritoneum; FDD, folate deficient diet; MeOH, methanol; Carb, Carbicarb; PBS, phosphate buffered saline; mPEG, mono methoxy polyethylene glycol.

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the toxic metabolite responsible for the ocular toxicity in methanol-poisoned humans [7].

Formate dehydrogenase (EC 1.2.1.2) acts directly by converting formate into CO_2 in the presence of NAD. Efficacy of single bolus intravenous administration of native formate dehydrogenase, isolated from the *Candida boidinii*, in detoxification of formate, in methanol poisoning was reported in our earlier study [8].

Chemical modification of therapeutic proteins with mono methoxy poly ethylene glycol (mPEG) to reduce the immunogenicity and to increase the circulating half life has been extensively studied [9–11]. Asparaginase modified with mPEG, is accepted by FDA, in patients with immunological reaction towards the free enzyme therapy [12]. In this study, formate dehydrogenase was modified with linear *N*-hydroxysuccinimidyl ester of mono methoxy polyethylene glycol propionic acid (mPEG-SPA 5000). The associated changes such as immunogenicity, allergenicity, plasma half life were evaluated and compared with that of native enzyme.

Rodents develop formate accumulation and acidosis similar to human beings in methanol poisoning, only when they are made folate deficient [13]. In vivo efficacy of PEG–FD or native FD was comparatively evaluated by single bolus intravenous administration of PEG–FD or native FD in folate deficient methanol intoxicated albino rats, along with Carbicarb buffer infusion. Carbicarb was used to treat the metabolic acidosis. Carbicarb is an alkalinizing agent, combination of 0.33 mol/l NaCl and 0.33 mol/l sodium bicarbonate [14–17]. Carbicarb besides correcting the metabolic acidosis which develops due to formate accumulation, provides a favorable pH environment for the enzyme to act. It has been reported in our previous published work [18]. Minimum optimal pH of formate dehydrogenase enzyme is 7.5 [19].

2. Materials and methods

2.1. Reagents

mPEG-SPA (mol.weight 5000) was from Fluka chemicals. Formate dehydrogenase (10.5 u/mg protein) from *C. boidinii*, Sephacryl 300 HR, and DEAE Sepharose FF were from Sigma-Aldrich Co, St. Louis, MO. Rabbit Anti-Rat IgG HRP conjugated secondary antibody was from Bangalore Genei, India. All other reagents were of analytical grade. Formate dehydrogenase enzyme has a molecular weight of 74 Kda [19]. The enzyme exists as a homodimer of 42 kDa subunits each possessing an independent active centre, and has no apparent metal-ion requirement [20].

2.2. Animals

Male Wistar rats (150–170 g) were used. Animals were maintained in a clean rodent room. They were housed 2–3 per cage in cages that were fitted with stainless-steel wire-mesh bottoms. They were maintained at a temperature of 28 ± 1 °C, and under a daily photoperiod of 12-h light/dark cycle. The animals were fed with pellet diet (Hindustan Lever Ltd., Mumbai, India) and tap water ad libitum. The animals were handled according to the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Govt. of.India. The experimentation protocol was reviewed and approved by Institutional Ethical committee.

2.3. PEGylation reaction

PEG reagents were dissolved in 20 mmol/l sodium phosphate buffer (pH 8.3) at concentration between 10 and 40 mmol/l. Initial reactions were carried out at molar ratios of PEG to protein between 40:1, 80:1 and 120:1 ratio to determine the optimal conjugation conditions and monitored on SDS-PAGE. The ratio of 40:1 was then used for all subsequent reactions.

PEGylation reactions were carried out in reaction buffer (0.2 mol/l sodium phosphate buffer, pH 8.3), at 20 °C for 30 min. The reaction buffer also contains 10 mmol/l sodium formate which is the substrate of the enzyme. The reactions were stopped with one-tenth volume of stop buffer (1 mol/l sodium phosphate buffer, pH 6.5) at 0 °C [21]. PEG–FD was then concentrated with Amicon Ultra-4 (10 kDa cut off) centrifugal filter unit.

2.4. Purification of PEG–FD

2.4.1. Sephacryl S-300 HR

Unreacted PEG was removed by size-exclusion chromatography [21]. Size exclusion column (30 cm × 1 cm diameter) was packed with pre swollen Sephacryl S-300 HR (75–125 μm) micro beads purchased from Sigma Chemicals Pvt. Ltd, USA. Five milliliters of PEGylation products (1 mg/ml) were loaded on the column which was equilibrated and eluted with 0.15 mol/l sodium chloride in 10 mmol/l sodium phosphate (pH 7.4) at a flow rate of 1 ml/min.

2.4.2. DEAE Sepharose FF

The un-PEGylated FD was separated from PEGylated FD by DEAE Sepharose FF column chromatography [21]. Anion exchange column (30 cm × 1 cm diameter) was prepared by using the DEAE Sepharose micro beads (25–45 μm) from Sigma Chemicals Pvt. Ltd. The column was equilibrated with equilibration buffer (50 mmol/l NaCl in 10 mmol/l sodium phosphate pH 7.4) at 1 ml/min flow rate.

2.4.3. Enzyme activity of PEG–FD

PEG–FD activity was measured in a mixture of 150 μmol potassium phosphate buffer pH 7.5, 5 μmol NAD, 500 μmol formate and limiting amounts of enzyme in a total volume of 3 ml. The increase in absorbance at 340 nm was read. [19]. One unit of enzyme was defined as the amount of enzyme that catalyzes the conversion of one micromole of NAD to NADH per minute at pH 7.5 at 37°C.

2.4.4. Serum half life of PEG–FD

In vivo serum clearance rates for both native FD and PEG–FD (40:1) were analyzed in 2 groups of albino rats, each consists of five rats (180–200 g). Purified PEG–FD or native FD (150 units) was injected into the left jugular vein. Blood was collected from the right jugular vein at the following time points namely before injection, 5, 15, 30, 45 min, 1, 2, 4, and 8 h after injection. Enzyme