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Preparation of monoclonal antibodies against a derivative of semicarbazide as a metabolic target of nitrofurazone

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Abstract

Monoclonal antibodies (McAb) were produced to detect semicarbazide (SEM), a metabolite as a marker residue of nitrofurazone in animal food production. A carboxyphenyl derivative (CPSEM) of SEM was synthesized following derivatisation with 4-carboxybenzaldehyde (CBA). CPSEM was purified by recrystallization and conjugated to bovine serum albumin (BSA) or ovalbumin (OVA) as immunogen or coating antigen, respectively. Hybridomas were obtained by fusing mouse myeloma cells SP2/0 with splenocytes from the mice immunized with CPSEM–BSA. Hybridomas 1F10 and 4F2 secreting antibodies against CPSEM were obtained and subcloned. Ascites of monoclonal antibodies were prepared by injecting 1×10^6 cells of hybridoma 1F10 into mice abdomen. McAb obtained from hybridoma 1F10 was highly specific for CPSEM and had no cross-reaction with various nitrofuran metabolites and a range of veterinary drugs. The sensitivity of the McAb to SEM was 0.01 ng mL^{-1} and the IC$_{50}$ value was 1.3 ng mL^{-1} (SEM in the form of CPSEM). McAb 1F10 is suitable to develop an immunoassay for SEM with sufficient sensitivity for monitoring nitrofurazone residues.

Keywords: Monoclonal antibodies; Semicarbazide; Nitrofurazone; Nitrofurazone metabolites

1. Introduction

Nitrofurazone is a broad-spectrum anti-bactericidal drug and belongs to the class of nitrofuran. It also possesses some anti-protozoal activity and is used both therapeutically and prophylactically in a number of food-producing animal species including pigs, sheep, goats, cattle, chickens and turkeys as a growth promoter [1]. Because nitrofuran drugs have potentially harmful effects on human health, the European Union has prohibited their use in food-producing animals [2].

Nitrofuran drugs are rapidly metabolized in vivo and do not persist as residues of the parent drugs in edible tissues [3,4]. The metabolites of these drugs bind to tissue proteins and persist for considerable periods in animal tissues after treatment and the protein-bound residues are stable in tissue matrices, even after long-term storage [5–7]. In the case of the nitrofuran drugs furazolidone, furaltadone, nitrofurantoin and nitrofurazone, a proportion of the bound metabolites possess the intact side chain 3-amino-2-oxazolidone (AOZ), 5-morpholino-3-amino-2-oxazolidone (AMOZ), 1-aminohydantoin (AHD) and semicarbazide (SEM), respectively [8]. These residues may be released as potentially toxic entities in the acidic conditions of human stomach [9].

SEM is a metabolite of nitrofurazone and has been used as a marker residue for illegal use of this drug in animal food production [10,11]. However, SEM in food may originate from other sources in contact with plastics foamed with the blowing agent azodicarbonamide (AZDC) [12,13]. From August 2005, the European Union would no longer permit the use of azodicarbonamide as a blowing agent in food contact materials [14]. SEM belongs to a family of chemicals (hydrazines) that are known to cause cancer in laboratory animals. While SEM has not been extensively tested for toxic effects, it may also be genotoxic and has been reported to be a weak animal carcinogen [15–18].

Various methods have been published for the determination of SEM by liquid chromatography electrospray ioniastion tandem mass spectrometry (LC-ESI/MS/MS) [19], liquid chromatography electrospray ionisation mass spectrometry (LC–MS)

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[20] and liquid chromatography/mass spectrometry/mass spectrometry (LC–MS/MS) [21–24]. However, with the advent of testing the third-world country imports and meat production in the EU, there is the urgent need for rapid, high throughput screening methods for tissue-bound residues of the nitrofurans. Currently, there is no report on the methods of immunoassay-based screening test for SEM of the tissue-bound nitrofuran residues and preparation of antibody against SEM except AOZ [25–27].

In this study, we report the preparation of monoclonal antibodies capable of detecting SEM following derivatisation with 4-carboxybenzaldehyde. These antibodies are suitable to develop an enzyme-linked immunosorbent assay (ELISA) for screening test of SEM.

2. Materials and methods

2.1. Materials

All chemicals, unless otherwise stated, were of analytical reagent grade. Semicarbazide hydrochloride and urea hydrogen peroxide addition were obtained from Sigma-Aldrich (Germany). Bovine serum albumin (BSA), ovalbumin (OVA), 4-carboxybenzaldehyde (CBA) and *N*-hydroxysuccinimide (NHS) were purchased from Sigma-Aldrich (Japan). Dicyclohexylcarbodiimide (DCC) and Freund's adjuvant (complete and incomplete) were obtained from Sigma-Aldrich (St. Louis, USA). Hypoxanthine/thymidine/aminopterin (HAT) and hypoxanthine/thymidine (HT) were bought from Sigma. RPMI-1640 with L-glutamine was obtained from Gibco. Polyethylene glycol 1500 (PEG 1500, 50%) was from Roche Diagnostics Corporation (Indianapolis, USA). Fetal calf serum was from TBD Biological technology Co. Ltd. (Tianjing, China). Peroxidase-labelled goat anti-mouse IgG (H + L) (HRP-IgG) was obtained from Jackson Immunoresearch Laboratories Inc., USA. Microtiter plates were supplied by Greiner Bio-one (Germany).

Cell culture plates (24 and 96 wells) and culture flasks were obtained from Costar Inc. (Cambridge, USA). As an ELISA equipment, microtiter plates (Costar Inc., Cambridge, USA), a 12-channel pipette (50–300 μL, Thermo Labsystems Co. Ltd., Shanghai, China) and a microplate reader (synergy-HT, BIO-TEK, USA) were used. Water was obtained from a MilliQ purification system (Millipore). LC/MS/MS system model API 3000 was used to measure the derivative of SEM (CPSEM) (Applied Biosystems, USA).

2.2. Buffer and solution

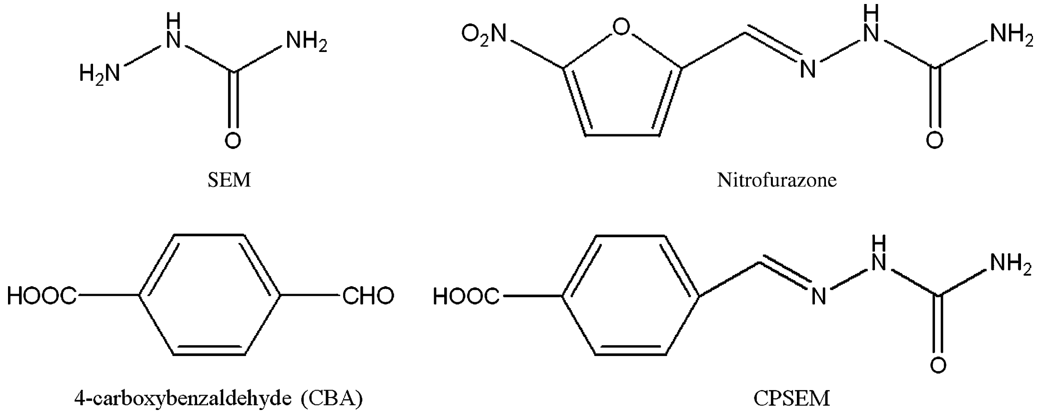
The following buffers were used: (1) stock solution of CPSEM at a concentration of 1 mg mL^{-1} was prepared in dimethylformamide and diluted to standard solution with PBS; (2) coating buffer was 0.1 M carbonate buffer with pH 9.6; (3) block buffer was 1% OVA (m/v) in PBS; (4) phosphate buffer saline (PBS) with 0.8% (m/v) NaCl, pH 7.2; (5) PBST was PBS buffer containing 0.05% Tween 20 (v/v) for washing; (6) substrate buffer was 0.1 M citrate, pH 5.5; (7) substrate solution for the peroxidase enzyme was prepared by mixing 200 μL 1% (w/v) TMB in DMSO with 64 μL 0.75% (w/v) to 20 mL substrate buffer; and (8) the enzymatic reaction was stopped with 2 mol L^{-1} H_2SO_4.

2.3. Synthesis of SEM derivative

An excess of SEM over CBA was stirred overnight in pyridine. Thin-layer chromatography of the reaction mixture demonstrated formation of a product (elution in 10% methanol in chloroform). After removal of pyridine by evaporation, the resulting mixture was crude CPSEM. The pure CPSEM was obtained by recrystallization from dimethylformamide. The chemical structures of SEM, CPSEM and nitrofurazone are shown in Fig. 1. The derivative of SEM was confirmed by LC/MS/MS.

2.4. Preparation of CPSEM complete antigen

CPSEM (Fig. 1) was conjugated to an immunogenic carrier protein BSA or OVA via an active ester method [28,29]. The carboxylic acid on the hapten was activated with DCC and NHS to produce an active ester, which then reacts with amine groups on BSA or OVA to form an amide bond (Fig. 2).



SEM Nitrofurazone

4-carboxybenzaldehyde (CBA) CPSEM

Fig. 1. Chemical structures of nitrofurazone, its marker residue SEM and the cross-linking hapten CPSEM.