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Identification and analysis of an impurity inducing clinical adverse effect in anti-adhesion carboxymethyl chitosan products☆

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ABSTRACT

Controlling and minimizing the adverse effects of drugs are the key issues in ensuring the safety of drug therapy. Carboxymethyl chitosan has been widely used as an anti-adhesion material. However, recently in China there have been several reported instances of conjunctival hyperemia associated with the use of carboxymethyl chitosan containing products. Through MS, FTIR, and GC analysis, an impurity, diglycolic acid, was discovered in carboxylmethyl chitosan products. Pharmacological tests further indicated diglycolic acid has antithrombogenicity properties and induces vasodilation, both of which can cause conjunctival hyperemia. Thus, through these tests it was ascertained that diglycolic acid was the culprit responsible for the adverse clinical effects. Next, emphasis shifted to trying to discover the mechanism responsible for generating the diglycolic acid. Under strong basic conditions, chloroacetic acid can generate glycolic acid, which, upon etherification, can become diglycolic acid. In order to avoid future adverse effects, we have established an HPLC method to detect and determine diglycolic acid in carboxymethyl chitosan products. This method is specific, accurate, and precise, and can be easily implemented into routine monitoring practice. Concurrently, a refined method has also been established in order to eliminate diglycolic acid from carboxymethyl chitosan.

1. Introduction

The safety and efficacy of pharmaceuticals are two fundamental requirements in drug therapy. Controlling impurity in pharmaceutical products is among the primary goals in the drug development [1]. The impurities in drugs often cause unwanted side effects by which the benefit from their curative effect could be outweighed [2,3]. By estimating the impurities in the drug materials and establishing the security of the impurities, the danger can be minimized [4]. Therefore, monitoring and controlling of impurities are very important in the assurance of the quality and safety of the drugs [5].

Postsurgical adhesions, as a natural part of the body's healing process, often occur after clinical surgeries, especially obstetric, gynecologic, cardiothoracic, and orthopedic surgery, due to the formation of fibrous bands forming between tissues and organs [6,7]. Things can be even worse in peritoneal adhesions, which can lead to complications such as intestinal obstruction, infertility, and chronic pelvic pain [8]. According to the latest report, up to $1.3 billion was spent in the U.S. annually for adhesion-related problems [9].

Currently, the major ways used to prevent adhesions include improvement of surgical methods, drug therapy, biological therapy, and the use of spacers [10]. Among them, using anti-adhesion materials as a spacer is thought to be effective. The ideal anti-adhesion material should be nontoxic, absorbable, capable of being fixed into position, easy to apply, and have good biocompatibility [11,12]. Recently, many polymer materials with high biocompatibility and absorption, such as hyaluronic acid, cellulose, and chitosan and its derivatives, have been used as anti-adhesion materials [13,14]. Carboxymethyl chitosan (CMC), prepared by the carboxymethylation of chitosan, is a water-soluble derivative of chitosan. CMC holds enhanced biological and physicochemical properties compared to chitosan and is a promising drug candidate for biomedical applications [15]. Carboxylmethyl chitosan has been used for wound healing [16], tissue engineering [17], drug delivery [18], targeted drug delivery [19], gene therapy [20], and biosensor imaging [21]. The use of CMC as an anti-adhesion material has been reported in some papers, Krause [22], Zhou [23], and Keenedy [24] used the N,O carboxymethyl chitosan (NOCC) hydrogel as the anti-adhesion material, and the results showed that NOCC can significantly reduce adhesions without producing side effects.

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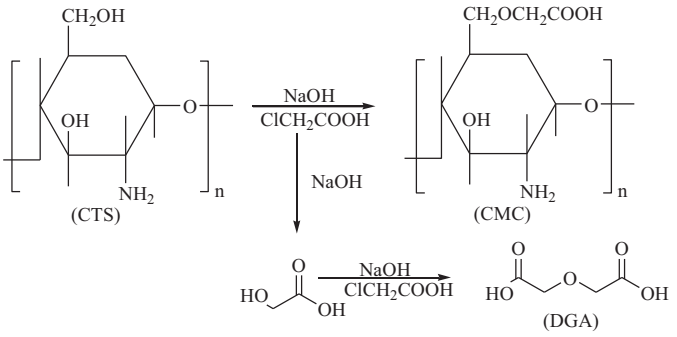

Fig. 1. The scheme of synthesis of O/N-carboxylated chitosan and conversion of glycolic acid and diglycolic acid.

Anti-adhesion products composed of carboxymethyl chitosan and saline were approved by the Chinese Food and Drug Administration (CFDA) in 2003. However, in September 2008, the CFDA began receiving reports of conjunctival hyperemia caused by the use of CMC anti-adhesion products. Subsequently, the CFDA recalled all the CMC products and requested all companies to stop production to re-evaluate the security of their products [25]. There was much urgency to find the reason behind the adverse effects caused by the CMC products. Hence, our lab has focused on finding the reasons for the conjunctival hyperemia in cooperation with the Saikesaisi pharmaceutical Co., Ltd. of Shandong since September, 2008. According to our studies, an impurity, diglycolic acid, which was found in CMC products, causes the adverse effect. Based on these findings [26], CFDA required companies to develop an analytical method with which to detect the impurity in their products to meet the requirement of CFDA, and also issued a notice that the companies may resume production of CMC products in March and May, 2009 [27,28]. Since then, CFDA has not received any adverse effect reports concerning CMC products.

In this paper we describe how we identified the impurity in carboxymethyl chitosan containing products, how we developed an analytical method to detect it using HPLC, and the mechanism of how this impurity induces the adverse symptoms.

2. Materials and methods

2.1. Sample, reagents, and animals

Carboxymethyl chitosan samples: A (080701) and B (080501), were obtained from Saikesaisi Pharmaceutical Co., Ltd. of Shandong (Shandong, China). Sample C (080602) was obtained from a company in Qingdao (Shandong, China). Anti-adhesion gel samples: D (071102) and E (080710) were obtained from Saikesaisi pharmaceutical Co. Ltd. of Shandong (Shandong, China). Sample F (20080702) was obtained from Shijiazhuang Yishengtang Medical Supplies Ltd. (Hebei, China). Diglycolic acid was obtained from Sigma–Aldrich (St. Louis, MO, USA) and the chemical structure was shown in Fig. 1. Methanol was HPLC grade. All other chemicals and reagents used were of analytical grade.

New Zealand white rabbits (2.5–3.0 kg) were purchased from Lukang Pharmaceutical Group Co. Ltd. of Shandong (License No. SCXK(LU)20050017).

2.2. Impurity characterization

The samples were analyzed using mass spectrometry on a Q-TOF Global mass spectrometer with ESI source (Waters Corporation, United States). The infrared spectra were taken on a Nicolet Nexus 470 spectrometer (Nicolet, United States) using KBr pellet pressing method. GC chromatography was performed on Hewlett-Packard 5890 system with a 30 m × 250 μm × 0.25 μm AC-225 column (Agilent, United States) and flame ionization detector (FID).

2.3. The preparation of the derivatized impurity and the refined method for carboxymethyl chitosan

50 mg of sample was added to a methanol–water mixture (8:2, v/v, 1 ml), vortexed for 1 min, and extracted by ultrasound for 10 min. Then the samples were centrifuged for 10 min at 6000 rpm, the supernatant was derivatized with ethanol and concentrated sulfuric acid to produce its ethyl ester. The derivatised impurity was analyzed by GC, ESI-MS and infrared spectra.

1 g of raw carboxymethyl chitosan was added to 40 ml of 70% ethanol, and then stirred at 60 °C for 1 h, suction filtered, and the process was repeated until no chloride ions were present. The residue was dehydrated with absolute ethanol, dried at 105 °C to get the refined carboxymethyl chitosan.

2.4. Established HPLC methodology for monitoring impurities

2.4.1. Instrumentation and chromatographic conditions

HPLC analyses were performed on Waters 1525 system with a Symmetry300 C_{18} column (4.6 mm × 150 mm). The elution was isocratic with the mobile phase consisting of methanol–10 mM KH_2PO_4 (pH 2.5) (3:97, v/v). Flow rate was 0.6 ml/min and the column temperature was maintained at 30 °C. The injection volume was 20 μl with UV detection at 204 nm [29].

2.4.2. Extraction procedure

50 mg of the sample was added to a methanol–water mixture (8:2, v/v, 1 ml), vortexed for 1 min, and extracted by ultrasound for 10 min. The samples were centrifuged for 10 min at 6000 rpm and then diluted 10 times by the mobile phase before injection.

2.4.3. Method validation

The method was validated according to the Chinese Pharmacopeia requirements. The following validation characteristics were addressed: specificity, linearity, limit of detection, accuracy, and precision.

2.5. Coagulation test

Blood coagulation time (BCT) was calculated using a modified Lee–White method [30]. In brief, 1 ml of each sample was added to three glass tubes, and then incubated in a water bath at 37 °C. Blood was collected from the rabbit's carotid by using prepared syringes with 21-gauge needles. 2 ml of blood was transferred to glass tubes which had been previously incubated in a water bath. The tubes were kept in the water bath for 5 min, and then one of three parallel samples was selected at 30 s intervals to observe the blood coagulation by inclining the tube. We defined the blood as coagulated when blood flow was not observed despite 90° inclination of the tube. BCT was calculated as the time from the start of the blood collection until blood coagulation in the second tube [31–33].

2.6. Intracutaneous stimulation test

According to method of GB/T 16886.10, saline, samples D, E, diglycolic acid, and refined carboxymethyl chitosan were injected into the rabbits on both sides of rabbit's backbone by hypodermic injection. The points of injection on the sides of rabbit's backbone were observed after 24 h, 48 h, and 72 h.