Talanta 146 (2016) 483–489

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Preparation of polyclonal antibodies for nateglinide (NTG) and development of a sensitive chemiluminescent immunoassay to detect NTG in tablets and serum

Lei Zheng [a,1], Jing Wang [a,1], Jie Zhang [b,c,1], Zhaorui Song [a], Yaqing Dong [a], Yufen Wang [a], Zhongsheng Tong [b,c], Chuan Deng [a,d], Yongmei Yin [a], Meng Meng [a,*], Rimo Xi [a]

[a] College of Pharmacy, State Key Laboratory of Medicinal Chemical Biology and Tianjin Key Laboratory of Molecular Drug Research, Nankai University, Tianjin 300071, China
[b] Tianjin Medical University Cancer Institute and Hospital, National Clinical Research Center for Cancer, Tianjin, 300060, China
[c] Key Laboratory of Cancer Prevention and Therapy, Tianjin 300060, China
[d] Tianjin Sungene Biotech Co., Ltd., Tianjin 300450, China

ARTICLE INFO

Article history:
Received 8 June 2015
Received in revised form
1 September 2015
Accepted 6 September 2015
Available online 9 September 2015

Keywords:
Nateglinide
Polyclonal antibodies
Chemiluminescence immunoassay
Tablets
Serum

ABSTRACT

In this study, we prepared polyclonal antibodies against anti-diabetic drug nateglinide (NTG), and established a sensitive chemiluminescent immunoassay (CLIA) to detect NTG in tablets and serum. Two kinds of immunogens were synthesized using ethylcarbodiimide (EDC)/hydroxysuccinimide (NHS) and carbonyldiimidazole (CDI)/4-dimethylaminopyridine (DMAP) as coupling reagents respectively. When activated by EDC/NHS, more molecules of NTG coupled with carrier protein in immunogens. A horseradish peroxidase (HRP)-luminol-H_2O_2 system with *p*-iodophenol enhancement was applied in the CLIA analysis. The antibodies in EDC/NHS group showed higher titer, sensitivity and wider detection linear range than those in CDI/DMAP group, and were chosen for next studies. The developed CLIA assay exhibited good selectivity towards NTG among structually similar analogs. The method could detect as low as 0.35 ng mL^{-1} NTG in buffer, 2.1 ng mL^{-1} NTG in serum and 0.84 ng mL^{-1} NTG in tablets. The CLIA method provided consistent results with HPLC method (r=0.9986) in determination of NTG from 5.0 to 400 μg mL^{-1}. The CLIA method could detect 78 samples in one assay, and the samples need only dilution in pretreatment. As a summary, this research offers a sensitive assay for high-throughout screening of NTG in formulation control and pharmacokinetic studies.

1. Introduction

Nateglinide (NTG) is an anti-hyperglycemic agent for treatment of type-2 diabetes by stimulating the release of insulin from pancreatic β-cells through inhibition of potassium-ATP channels [1–3]. It is extensively biotransformed in liver via cytochrome P450 isoenzymes CYP2C9 and CYP3A4. The major metabolites of NTG are less potent in anti-diabetic effect than NTG [3]. NTG has fast onset of action with t_{max} in 0.92–1.31 h. Thus, it is usually administrated 15 min before a meal. When NTG was orally administered at a dose of 120 mg, a peak serum concentration (C_{max}) is measured to be 10.0–15.0 μg mL^{-1} [4, 5]. Its hypoglycemic effect should be regularly monitored for patients with severe heart disease, damaged liver or kidney functions, and patients administered with other kinds of anti-diabetic drugs at the same time [6].

Only a few methods have been developed that focus on the identification and determination of NTG in pharmaceutical formulations and biological matrices. A great effort was performed to reduce the limit of detection (LOD) of chromatography methods for NTG. Accordingly, RP-HPLC–UV (LOD=60 ng mL^{-1}) [7], LC–MS/MS (LOD=20 ng mL^{-1}) [8], stereoselective HPLC (LOD=200 ng mL^{-1}) [9], LC-ESI–MS (LOD=50 ng mL^{-1}) [10], HPTLC (LOD=200 ng mL^{-1}) [11], micellar liquid chromatography (LOD=800 ng mL^{-1}) [12], spectrofluorimetric determination (detection linear range: 50–500 ng mL^{-1}) [13] have been developed for NTG assay. The pretreatment procedure for biological samples usually needs deproteinization, extraction for separation, dryness then reconstitution for test, and the methods are not sensitive enough.

As a contrast, immunoassays show obvious advantages in convenient operation and low cost. To our knowledge, there is no report about development of antibodies and immunoassays for

* Corresponding author. Fax: +86 22 23507760.
E-mail address: mengmeng@nankai.edu.cn (M. Meng).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.talanta.2015.09.008
0039-9140/

NTG. In this work, we used ethylcarbodiimide (EDC) and carbonyldiimidazole (CDI) as coupling reagents and synthesized two types of NTG immunogens. Coupling ratio of NTG with carrier proteins was evaluated. Titer, sensitivity and detection range were measured for each group of NTG antibody. With the optimal antibody product, horseradish peroxidase (HRP)-luminol-H_2O_2 system was developed with *p*-iodophenol as an enhancer. Under alkaline conditions, luminol could be oxidated by HRP to form 3-aminophthalate ion [14]. The proposed CLIA exhibited a significantly higher sensitivity than traditional enzyme-linked immunosorbent assay (ELISA), and showed great potential in high-throughput screening of NTG in pharmaceutical formulations and serum.

2. Materials and methods

2.1. Chemicals and apparatus

Nateglinide (NTG) and repaglinide were purchased from the Institute for Chemical Drug Control (National Institutes for Food and Drug Control, Beijing, China). Glimepiride, rosiglitazone, metformin, prazosin and acarbose were from Dalian Meilun Biotech Co., Ltd. (Dalian, China). Bovine serum albumin (BSA), ovalbumin (OVA), 1-(3-dimethylaminopropyl)ethylcarbodiimide hydrochloride (EDC.HCl), *N*-hydroxysuccinimide (NHS), ethylenediamine, 4-dimethylaminopyridine (DMAP), *N,N′*-carbonyldiimidazole (CDI), 3,3′,5,5′-tetramethylbenzidine (TMB), 2,4,6-trinitrobenzenesulfonic acid (TNBS), Freund's incomplete adjuvant, Freund's complete adjuvant, *p*-iodophenol, 5-amino-1,2,3,4-tetrahydrophthalazine-1,4-dione (Luminol) were purchased from Sigma-Aldrich (St. Louis, MO). Goat anti-rabbit IgG labeled with HRP was from Tianjin Sungene Biotech Co., Ltd. (Tianjin, China).

CLIA intensity was measured by Hamamatsu BHP9504-Microplate Luminometer (Hamamatsu Photon Techniques Inc., Beijing, China). ELISA signal was recorded by enzyme immunoassay microplate reader Model 680 from Bio-Rad Laboratories Headquarters (Bio-Rad Laboratories Co., Ltd, Shanghai, China). Polystyrene microtiter plates (96-well) were from Jet Bio-filtration Products, Co., Ltd. (Jet Biofil, Beijing, China).

Table 1
Coupling ratio of immunogens and coating antigen.

Sample	ε -amino groups	Coupling ratio[a]
BSA	59	–
cBSA	65.8	–
NTG-EDC-cBSA	43.5	22.3:1
NTG -CDI-cBSA	57.6	8.2:1
OVA	20	–
cOVA	31.9	–
NTG-CDI-cOVA	22.2	9.7:1

[a] Coupling ratio is the molar ratio of hapten to carrier protein.

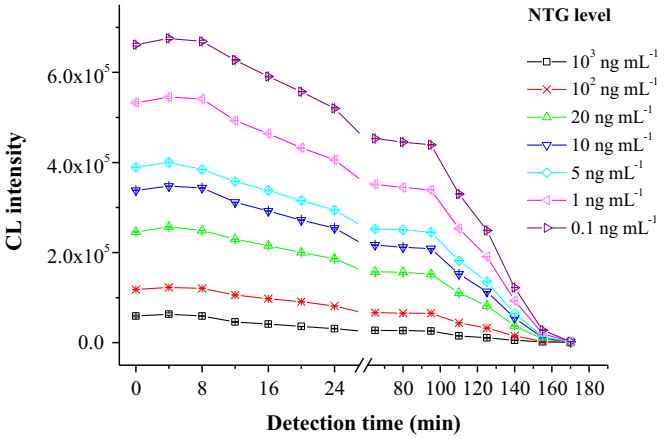

Fig. 2. Kinetics of chemiluminescence reaction of the CLIA method in determination of NTG with different concentration.

2.2. Solutions

(1) Phosphate buffered saline (PBS, pH7.4): 138 mmol L^{-1} NaCl, 1.5 mmol L^{-1} KH_2PO_4, 7 mmol L^{-1} Na_2HPO_4 and 2.7 mmol L^{-1} KCl; (2) Washing buffer (PBST): a PBS solution containing 0.05% (v/v) of Tween 20; (3) Coating buffer (pH9.6): 0.05 mol L^{-1} carbonate buffer containing 15 mmol L^{-1} Na_2CO_3 and 35 mmol L^{-1} $NaHCO_3$; (4) Blocking buffer: PBS mixed with 1% of OVA and 0.05% (v/v) Tween 20; (5) TMB solution for ELISA test (pH5.6): 1 mmol L^{-1} TMB mixed with an equal volume of 1 mmol L^{-1} urea hydrogen peroxide (H_2O_2) citrate buffer; (6) Luminol solution for

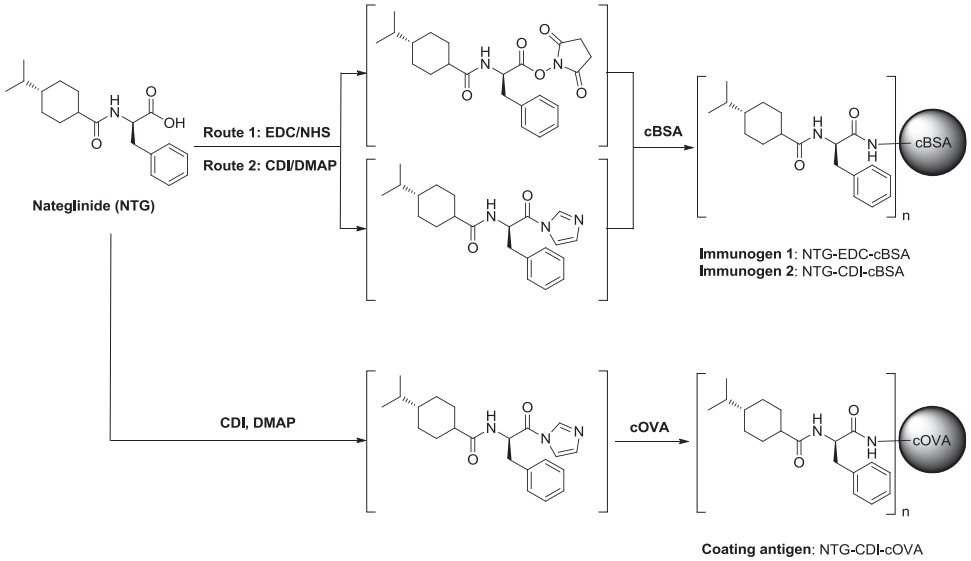

Fig. 1. Synthesis of immunogens and coating antigen for nateglinide (NTG) using ethylcarbodiimide (EDC)/hydroxysuccinimide (NHS) and carbonyldiimidazole (CDI)/4-dimethylaminopyridine (DMAP) as coupling reagents, respectively.