Anal Bioanal Chem (2015) 407:1973–1980
DOI 10.1007/s00216-015-8462-3

RESEARCH PAPER

A sensitive chemiluminescent immunoassay for point-of-care testing of repaglinide in natural dietary supplements and serum

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Received: 11 October 2014 / Revised: 23 December 2014 / Accepted: 5 January 2015 / Published online: 6 February 2015

Abstract For point-of-care testing of the illegal fortification of repaglinide (Rep) in natural dietary supplements, a competitive chemiluminescent immunoassay (CLIA) was established, using a horseradish peroxidase (HRP)–luminol–H_2O_2 system for signal amplification. Polyclonal antibodies for Rep were produced via immunization technique. Following optimization of the enzyme reaction time and concentrations of antibody and coating antigen, the method showed a limit of quantification (LOQ) of 1.0 ng/mL in PBS and limit of detection (LOD) of 8.3 ng/mL in serum and 6.0 ng/mL in blank tablets. When applied in natural dietary supplements, the method provided results consistent with those from HPLC, suggesting that the proposed method could be used for rapid screening of Rep in natural dietary supplements and detecting Rep in serum after administration.

Keywords Repaglinide · Antibody · Chemiluminescent immunoassay · Natural dietary ingredients

Introduction

More and more "naturally sourced healthy ingredients (natural dietary supplements)" are commercially available for regulating blood glucose. The illegal fortification of chemical medicines in these formulations is a growing concern in most countries. On 16 March 2013, the State Food and Drug Administration in China (SFDA) implemented a regulation—"The list of chemical medicines illegally added in dietary supplements (1st batch)"—banning such products which were shown to contain hypoglycemic chemicals added in order to achieve significant antidiabetic effects.

Repaglinide (Rep, $C_{27}H_{36}N_2O_4$), a member of the carbamoylmethyl benzoic acid family [1], is recognized as a first-line mono-therapy for type 2 diabetic patients who have failed to respond adequately to diet alone [2, 3]. Without specific dosage, the spiked Rep in natural dietary supplements may be over-administered, which could lead to hypoglycemia with serious adverse effects including nausea, tiredness, perspiration, confusion, and even loss of consciousness [4]. Therefore, analytical methods for monitoring the addition of Rep in natural dietary ingredients and its plasma concentration after administration are urgently required.

Most determinations of Rep are still limited in instrumental analysis, e.g., reversed-phase thin-layer chromatography (RP-TLC; LOD=8 μg/mL) [5], HPLC (LOD=0.1 μg/mL) [6], RP-HPLC (LOD=0.1 μg/mL) [7], LC-MS-MS (LOD=0.01 ng/mL) [8, 9], and HPTLC (LOD=50 ng/spot) [10]. These methods show benefit in terms of good accuracy and repeatability; however, the disadvantages of high cost, time-consuming nature, and complicated pretreatment greatly limit their use in real-life situations, especially for point-of-care testing in clinical evaluation and food control.

Chemiluminescent immunoassay (CLIA) has been explored as a rapid analysis method in a wide range of applications owing to its extremely high sensitivity, simple instrumentation, and wide calibration ranges. As a result, it could be applied in pharmaceutical control, clinical diagnostics, and environmental monitoring [11, 12]. In this work, we synthesized an immunogen of Rep, produced its polyclonal antibody, and developed a CLIA for Rep in an

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indirect competitive format [13]. A horseradish peroxidase (HRP)–luminol–H_2O_2 system was used as the signal amplification strategy. To our knowledge, there is no report on immunoassay development for Rep, and this sensing system could be used for point-of-care testing of Rep in natural dietary supplements and serum.

Materials and methods

Apparatus and materials

Bovine serum albumin (BSA), ovalbumin (OVA), 1-(3-dimethylaminopropyl)ethylcarbodiimide hydrochloride (EDC·HCl), *N*-hydroxysuccinimide (NHS), ethylenediamine, 4-dimethylaminopyridine (DMAP), *N,N′*-carbonyldiimidazole (CDI), *N,N*-dimethylformamide (DMF), incomplete adjuvant (iFA), Freund's complete adjuvant (FA), sodium dodecyl sulfate (SDS), 4-iodophenol (PIP), 5-amino-1,2,3,4-tetrahydrophthalazine-1,4-dione (luminol), and trinitrobenzenesulfonic acid (TNBS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Repaglinide was purchased from the National Institutes for Food and Drug Control (Beijing, China). Nateglinide, glimepiride, rosiglitazone, metformin, prazosin, and acarbose were from Dalian Meilun Biotech Co., Ltd (Dalian, China). Goat anti-rabbit IgG labeled with HRP was from Sangon Biotech Co. (Shanghai, China). H_2O_2 (30 %, w/w) was from Tianjin Kermel Chemical Reagent Co., Ltd (Tianjin, China). Rep tablets were from Jiangsu Hansoh Pharmceutical Co., Ltd (Lianyungang, China). Two kinds of natural dietary supplements (capsule 1 and capsule 2) were purchased from a local drugstore (Tianjin, China).

Buffers and solutions: (a) sodium phosphate-buffered saline (pH 7.4) (PBS solution comprised 0.138 mol/L NaCl, 2.7 mmol/L KCl, 1.5 mmol/L KH_2PO_4, and 7 mmol/L Na_2HPO_4); (b) coating buffer (pH 9.6) (0.015 mol/L Na_2CO_3 and 0.035 mol/L $NaHCO_3$ in distilled water); (c) blocking buffer (10 g/L OVA in PBS containing 0.5 mL/L Tween 20); (d) washing buffer (PBST) (PBS buffer containing 0.5 mL/L Tween 20); (e) substrate buffer consisted of 0.5 mmol/L luminol, 0.2 mmol/L 4-iodophenol (PIP), and 0.1 ‰ (v/v) H_2O_2 (30 %, w/w).

UV data were from a UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan). The CLIA signal was recorded by a BHP9504-Microplate Luminometer (Beijing Hamamatsu Photon Techniques Inc., China). White polystyrene microplates used in CLIA performance were from Jet Biofiltration Products Co., Ltd. (Beijing, China).

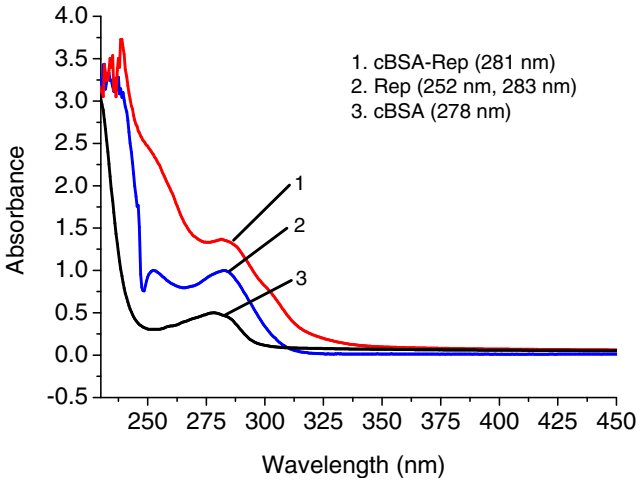


Fig. 2 UV spectra of Rep, cBSA, and immunogen (cBSA–Rep)

Preparation of immunogen and coating antigen

Carboxylic acid groups of the carrier proteins of BSA and OVA were converted to ε-amino groups by reaction with ethylenediamine to form cationized BSA (cBSA) and OVA (cOVA) [14].

Immunogen (cBSA–Rep) was synthesized as indicated in Fig. 1. Thus, 9.3 mg Rep, 39.5 mg EDC, and 11.8 mg NHS were dissolved in 2 mL of DMSO with stirring for 12 h at room temperature. The solution was mixed with 9 mL of PBS (0.01 M, pH 7.4) containing 40 mg cBSA and stirred at room temperature for 3 h. The mixture was dialyzed against PBS (0.01 M, pH7.4) for 3 days and in distilled water for another 3 days. The solution was lyophilized, and cBSA–Rep conjugate was stored at −20 °C. Coating antigen (cOVA–Rep) was prepared using CDI/DMAP as the coupling reagent in a similar way.

Production of polyclonal antibodies for Rep

Animal treatments were conducted with the approval of the Institutional Authority for Laboratory Animal Care. Two male New Zealand white rabbits were subcutaneously immunized

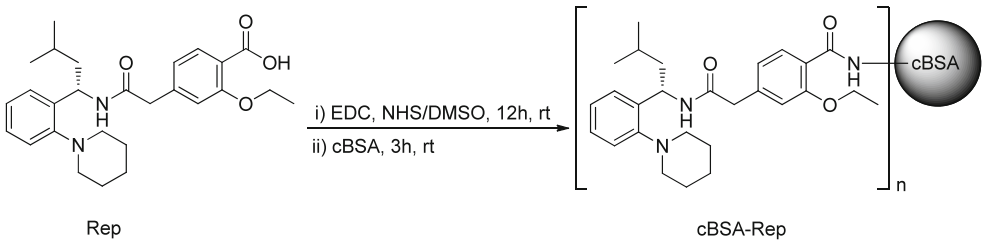


Fig. 1 Synthesis procedure for immunogen (cBSA–Rep)

Table 1 Coupling ratio of immunogen and coating antigen

Sample	Number of ε-amino groups	Coupling ratio[a]
BSA	59	–
cBSA	79.4	–
cBSA–Rep	32.4	47.1
OVA	20	–
cOVA	29.2	–
cOVA-Rep	25.2	4:1

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

for each immunogen. Before immunization, 1 mL of blood was taken as the negative serum. For the initial immunization, 0.5 mg immunogen in 0.5 mL of saline (0.9 %) was emulsified with 0.5 mL of FA and subcutaneously injected at multiple sites on the neck and back of the rabbits. Subsequent injections (0.25 mg immunogen in 0.5 mL saline mixed with 0.5 mL of iFA) were performed 3 weeks later. Immunizations were then performed three times at 2-week intervals. On the fifth immunization, 0.25 mg of immunogen was dissolved in 1 mL of saline without emulsification. One week later, all rabbits were exsanguinated by heart puncture after general anesthetic. The blood was allowed to clot for 2 h at room temperature, overnight at 4 °C, and then centrifuged at 8,000*g* for 10 min. The supernatant was collected and purified with 50 % (v/v) saturated ammonium sulfate (SAS). Impurities in the supernatant were removed, and antibody products in the precipitate were dissolved in physiological saline. The antibody in this mixture was further purified with 33 % (v/v) of SAS twice, as indicated above, to obtain the purified polyclonal antibody to Rep. Before analysis, the final precipitate of antibodies was dialyzed against PBS to remove ammonium sulfate.

CLIA performance

Wells in a microplate were coated with 100 μL of cOVA–Rep (0.5 μg/mL) at 37 °C for 2 h and then washed three times with washing buffer (250 μL/well). The unbound sites were then blocked by OVA in blocking buffer (200 μL/well) overnight at 4 °C. The wells were washed as indicated above and then 50 μL of analyte solution along with 50 μL of diluted Rep antibody solution was added and incubated at 37 °C for 0.5 h. The Rep in the analyte solution would also bind with Rep antibody, inhibiting the binding of coating antigen with Rep antibody. After the washing procedure, HRP-labeled goat-anti-rabbit IgG (1:4,000, 100 μL/well) was added and incubated for 30 min at 37 °C. After washing, 100 μL of chemiluminescent substrate was pipetted into each well, and CL intensity was recorded 5 min later.

CLIA application in natural dietary ingredients and serum

To decrease the effect of the matrix on the sensitivity of the CLIA method, blank serum and tablets were diluted to 1:50, 1:100, 1:500 or 1:1,000 with PBS, added with different concentrations of Rep, and tested by the CLIA. The dilutions with IC_{50} close to that in buffer were selected for the next steps.

Rep tablets and capsules were analyzed as these are the most commonly used formulations in natural dietary ingredients. Two Rep tablets were dissolved in 0.1 M HCl (100 mL), diluted to 80 ng/mL (Rep), and analyzed by CLIA. Granules in one capsule were dissolved in 0.1 M HCl (50 mL). The mixture was then centrifuged at 5,000*g* for 20 min and the supernatant was collected as a stock solution for future use.

Results and discussion

Verification of immunogen

Using active ester reagents, the carboxylic acid group of Rep was linked to cBSA and cOVA to form immunogen and coating antigen (Fig. 1). After dialysis, the conjugation products were analyzed by UV (Fig. 2). cBSA exhibited a characteristic absorption peak at 278 nm. Rep showed an absorption peak at

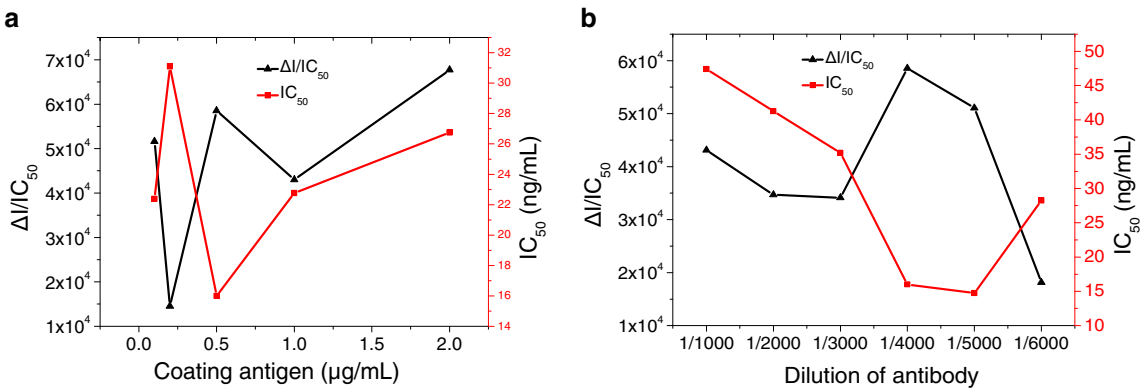


Fig. 3 Effect of concentration of coating antigen (**a**) and antibody (**b**) on $\Delta I/IC_{50}$ and IC_{50} value of CLIA test, where IC_{50} is the Rep level resulting in 50 % CL intensity decrease, and ΔI is the CL intensity for Rep solution of IC_{50} level minus that for blank group

283.5 nm, while cBSA–Rep showed peaks at 278 nm and 281 nm. A small shift was observed in the UV absorption peak of cBSA–Rep, which is probably from a newly generated conjugate. The coupling ratio of hapten to carrier proteins was determined by a trinitrobenzenesulfonic acid assay (TNBS), based on reaction of ε-amino groups in carrier proteins with TNBS reagent. Since we know there are 59 TNBS reactive amino groups in BSA and 20 in OVA [15], the numbers of ε-amino groups in carrier proteins before and after conjugation could be calculated according to the standard curve (A=0.001316m+0.1773, R^2=0.9998), which was obtained by plotting the absorbance (A) at 335 nm versus the amount (m) of BSA (50–200 μg). As shown in Table 1, conjugation ratios are 47:1 for cBSA–Rep and 4:1 for cOVA–Rep.

Quantification of antibody

The concentration of polyclonal antibody for Rep was quantified by the Lowry–Kalckar formula: protein concentration (mg/mL)=(1.45A_{280}−0.74A_{260}) × dilution ratio of polyclonal antibodies. A_{280} and A_{260} represent the absorbance of polyclonal antibodies at 280 nm and 260 nm, respectively. As a result, the concentration of the polyclonal antibodies was determined to be 12.06 mg/mL.

Optimization and characteristics of CLIA

Concentration of coating antigen and Rep antibody

The sensitivity of an immunoassay mostly depends on the amount of immunoreaction complexes [16]. Therefore, the concentrations of Rep antibody and coating antigen were optimized. Firstly, antibody dilution was applied at 1:2,000 and

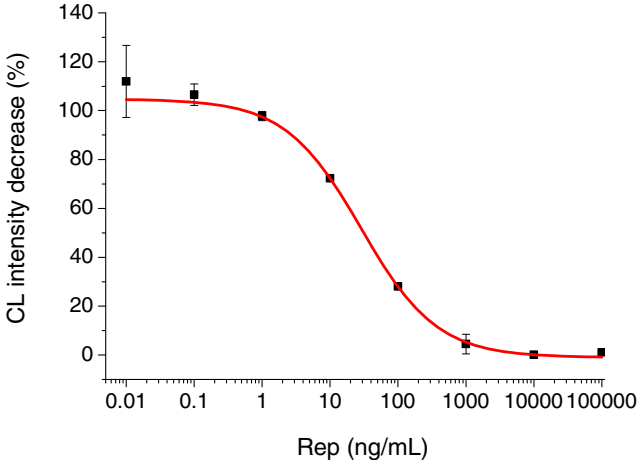


Fig. 5 Inhibition curves of CLIA method to detect Rep (n=3)

the concentration of coating antigen ranged from 0.1 to 2 μg/mL. A lower value of IC_{50} with higher $\Delta I/IC_{50}$ indicates higher sensitivity, where IC_{50} is the Rep level resulting in a 50 % CL intensity decrease, and ΔI is the CL intensity for Rep solution of IC_{50} level minus that for blank group. As was indicated in Fig. 3a, 0.5 μg/mL of coating antigen was chosen. Then, 0.5 μg/mL of coating antigen was selected, while different dilutions of Rep antibody were applied. A low value of IC_{50} was obtained when the Rep antibody was diluted at 1:4,000 and 1:5,000, while $\Delta I/IC_{50}$ is much higher for a dilution factor of 1:4,000 (Fig. 3b). Finally, 1:4,000 was selected as the optimal dilution factor for the higher intensity increase.

Time for enzyme reaction

After the CL substrate buffer was added, the CL intensity increased, achieved a stable period, and finally decreased.

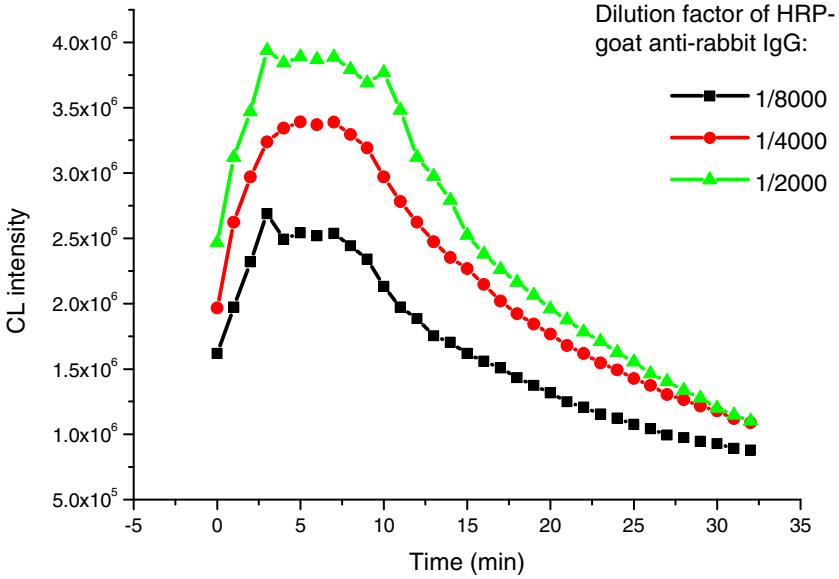


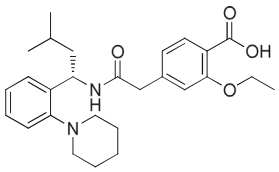
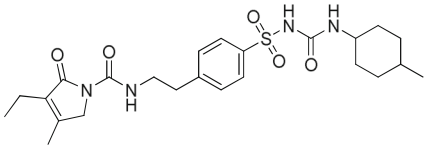
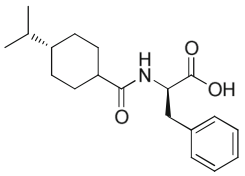
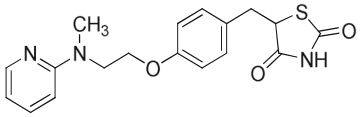
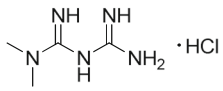
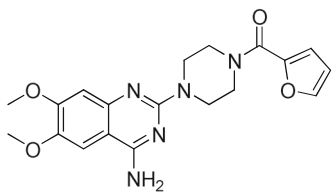
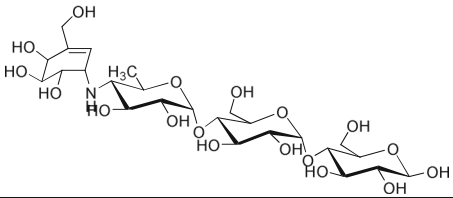
Fig. 4 CL kinetic curves using different diluted HRP-labeled goat-anti-rabbit IgG

The reaction time required to achieve a strong and constant signal is favorable for improving sensitivity and stability. CL kinetic curves were plotted by measuring the CL intensity every minute after adding the substrate. For three diluted factors of HRP-labeled goat-anti-rabbit IgG, the CL intensity increased quickly before 4 min, stayed constant for several minutes after 5 min, then gradually decreased (Fig. 4). Therefore, we selected 5 min as the optimal time for the enzyme reaction.

Sensitivity and specificity

Under the optimal conditions, a typical competitive inhibition curve was obtained between CL decrease percentage and Rep concentration (C) (Fig. 5). IC_{50} value was calculated to be 28.58 ng/mL according to the midpoint of the curve. The CL intensity decrease was linearly related to Rep with the concentration from 1 to 100 ng/mL (R=0.9983). The results

Table 2 Cross-reactivity of CLIA to related compounds

Analyte	Structure	IC_{50} (ng/mL)	Cross-reactivity (%)
Repaglinide		28.58	100%
Glimepiride		>100000	<0.029%
Nateglinide		>100000	<0.029%
Rosiglitazone		>100000	<0.029%
Metformin		>100000	<0.029%
Prazosin		>100000	<0.029%
Acarbose		>100000	<0.029%

indicated that as low as 1.0 ng/mL (limit of quantification, LOQ) Rep could be sensitively detected using this method.

We then investigated the specificity of the method for Rep by testing one structural analogue (nateglinide) and five functional analogues (glimepiride, rosiglitazone, metformin, prazosin, acarbose). The IC_{50} value was measured for each analyte, and the cross-reactivity (CR) for each analogue was calculated according to the following equation:

$$CR(\%) = (IC_{50}\,\text{of Rep}/IC_{50}\,\text{of analogue}) \times 100\%$$

As indicated in Table 2, none of these analogues showed obvious binding with Rep antibody (<0.01 %), even at a 300-fold higher concentration than that for Rep. These observations demonstrated that the proposed CLIA method exhibits very high specificity for Rep.

Accuracy

A total of 102 samples in PBS containing 1.0–100.0 ng/mL Rep were determined by CLIA method. The detected data exhibited a good linear relationship with the known Rep concentration (R=0.9843 in 1.0–100.0 ng/mL, n=102; R=0.9500 in 1.0–25.0 ng/mL, n=47, Fig. 6a). The difference plot (Fig. 6b) versus the known values showed that 96 of the 102 samples had a difference between −27.98 % and 26.14 %, and 78 of the 102 samples had a difference between −14.45 % and 12.61 %, indicating that the proposed method is accurate [17].

After being administered, the concentration of Rep in serum may be much higher than the upper limit of the calibration curve, so the samples need to be diluted before analysis. The influence of dilution on the accuracy of the CLIA method should be studied. A Rep stock solution prepared in serum (320 ng/mL) was diluted by 2, 4, 8, 16, 32, 64, 128 and 256 fold and analyzed. As shown in Fig. 7, the detected Rep concentration showed excellent linear correlation with dilution

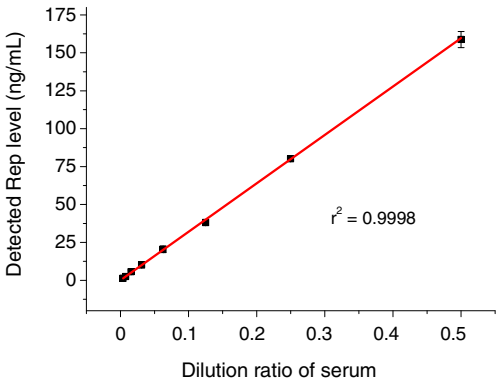


Fig. 7 Accuracy study of CLIA test in diluted serum (n=3)

factors and was also closely related to the calculated value. The results suggest that the proposed method could provide accurate quantification of Rep in serum which needed to be diluted before analysis.

CLIA application in natural dietary ingredients and serum

Rep solutions prepared in blank serum and tablets with different dilution factors were determined by the CLIA method. Finally, the blank serum diluted by 50-fold was selected for testing, and blank tablets could be directly determined without dilution.

Twenty blank serum and tablet samples were analyzed by the CLIA method to obtain an average detected Rep value (average) and standard deviation (σ). The limit of detection (LOD) of the method in each matrix was calculated to be LOD = average+3σ. As a result, the LOD value was 6.0 (i.e., 4.56+3×0.52) ng/mL in blank serum and 8.3 (i.e., 5.81+3×0.83) ng/mL in blank tablets.

According to LOD results, both matrixes were spiked with Rep (10, 25 and 50 ng/mL) and measured four times on 1 day and on three different days. As shown in Table 3, analytical

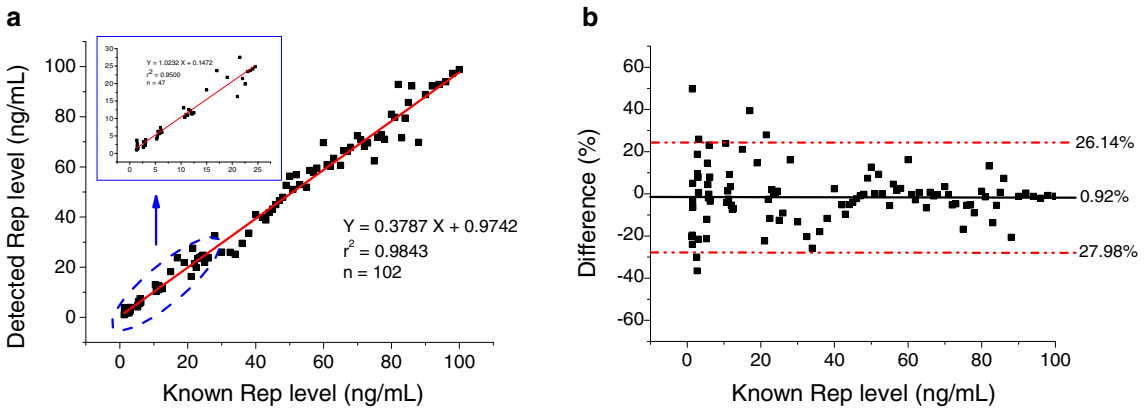


Fig. 6 **a** Correlation between results measured by the proposed CLIA and their known values. The *inset* shows the correlation at Rep concentration of 1.0–25.0 ng/mL. **b** Difference plot of 102 samples measured with the proposed CLIA. The *x-axis* represents the known Rep level, and *y-axis* represents $[100\times(Rep_{CLIA} - Rep_{calibrator})]/[(Rep_{CLIA} + Rep_{calibrator})/2]$. *Solid line* indicates the mean difference between the two methods. *Dashed lines* indicate the average difference value±2SD

Table 3 Recovery and coefficient of variation (CV) for Rep determination in serum and blank tablets by CLIA

Sample	Spiked (ng/mL)	Inter-assay			Intra-assay		
		Detected (ng/mL)	Recovery (%)	CV (%)	Detected (ng/mL)	Recovery (%)	CV (%)
Serum	10	11.5±1.4	115.6	11.9	10.4±0.9	104.7	9.1
	25	25.7±2.4	102.9	9.4	25.6±0.5	102.7	1.9
	50	53.5±2.1	107.1	3.8	49.7±3.3	99.4	6.7
Blank tablet	10	11.7±1.3	117.2	11.2	10.7±0.9	107.2	8.7
	25	24.3±1.4	97.2	5.7	25.2±0.8	101.1	3.4
	50	53.7±1.55	107.5	2.7	50.4±3.4	100.8	6.7

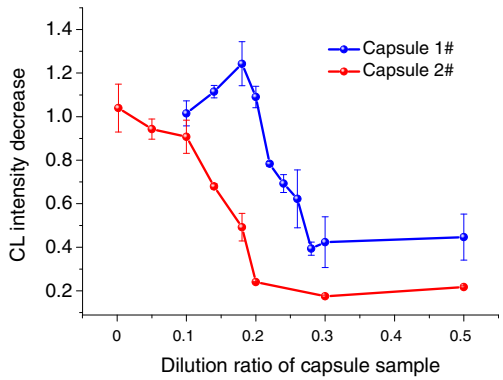


Fig. 8 CL intensity decrease in diluted capsule samples (n=3)

recoveries of Rep were 102.9–115.6 % and 99.4–104.7 % for inter- and intra-assay comparison in serum, respectively, and 97.2–117.2 % and 100.8–107.2 % for inter- and intra-assay comparison in blank tablets, respectively.

Capsules and tablets are the most commonly used formulations in products of natural diary supplements; hence, capsules (labeled amount of 0 mg Rep/capsule) and Rep tablets with a labeled amount of 1 mg Rep/tablet were analyzed. For method validation, these authentic samples were also measured by an HPLC method (an Inert Sustain C18 column 150×4.6 mm i.d., 5 μm, Shimadzu-GL, Shanghai, China), detected by an Ultimate 3000 liquid chromatography system at 240 nm (Dionex, Sunnyvale, CA, USA). The mobile phase was acetonitrile/

Table 4 Determination of Rep in authentic samples by CLIA and HPLC

Product	Labeled amount of Rep	CLIA		HPLC		Qualified?
		Found amount	CV (%)	Found amount	CV (%)	
Capsule 1	0 mg	6.65±0.82 μg/capsule	12.25	7.2±0.05 μg/capsule	4.12	No
Capsule 2	0 mg	24.32±1.70 μg/capsule	6.97	22.82±0.70 μg/capsule	3.04	No
Repaglinide tablet	1 mg/tablet	0.99±0.03 mg/tablet	2.47	0.98±0.04 mg/tablet	0.37	Yes

Table 5 Comparison of reported methods for Rep determination

Methods	Sensitivity/LOD	Linear range (ng/mL)	Matrix	Sample quantity	Assay duration (h)[b]	Reference
CLIA	8.3 ng/mL 6.0 ng/mL	1.0–100	Serum Tablets	78 [a]	1.25	This work
HPLC	10 ng/mL	20–200	Plasma	1	>1.5	[6]
TLC	8 μg/mL	60–360	Tablets	1	>1	[5]
RP-HPLC	0.1 μg/mL	2–35	Tablets	1	>1.5	[7]
LC-MS-MS	0.01 ng/mL	0.05–50	Plasma	1	>1.5	[9]
HPTLC	50 ng/spot	400–2,400 ng/spot	Tablets	1	>1.5	[10]

[a] On one microplate, 78 wells could be used to detect 78 samples, while the other 18 wells are used to establish the standard curve

[b] Assay duration included time for sample preparation, apparatus warming up (especially for instrumental analysis), and detection

ammonium formate (pH 2.7; 0.01 M) (60:40, v/v). The flow rate was 1.0 mL/min and the retention time of Rep was 8.3 min. Rep tablet was determined to contain 0.99±0.03 mg Rep/tablet when tested by CLIA. The result is consistent with that by the HPLC method (0.98±0.04 mg Rep/tablet). The detected amount by the two methods are both consistent with the labeled amount (1 mg/tablet), indicating that the proposed method could provide accurate quantification in Rep tablets.

Then, capsules of two kinds of commercial natural dietary supplements were assayed. The stock solution for each capsule was diluted and analyzed. The diluted samples leading to a 50 % decrease of CL intensity (3.85-fold for capsule 1 and 5-fold for capsule 2, Fig. 8) were chosen for analysis. The results detected by CLIA showed that the amount of Rep was 6.6±0.8 μg/capsule in capsule 1 and 24.3±1.7 μg/capsule in capsule 2; these results are in good agreement with those obtained by HPLC (Table 4), indicating the reliability of the CLIA, and thus suggesting that these two natural dietary ingredients were not qualified. In case of Rep detection, the proposed CLIA method shows comparable sensitivity with the published assays; however, the method could detect a large number of Rep samples on one microplate (Table 5) and therefore exhibits promising potential in the rapid screening of Rep.

Conclusion

In this work, we prepared sensitive and specific polyclonal antibodies for Rep and developed a CLIA to detect Rep in serum, Rep tablets, and products of natural dietary ingredients. The antibody showed a LOQ value of 1.0 ng/mL to Rep and little cross-reactivity towards other antidiabetic drugs. The limit of detection reached 8.3 ng/mL in serum and 6.0 ng/mL in tablets. The intra- and interassay coefficients of variation were both below 11.9 % when used in the serum and natural dietary supplements, and the determination is accurate according to a validation study with an HPLC method. In summary, the proposed assay could be used as a rapid screening method for clinical evaluation and quality control of natural dietary ingredients that might be illegally supplemented with Rep. The study is also relevant to the construction of rapid analyses of other chemicals that may be added to natural dietary ingredients.

Acknowledgment This work was received financial support from the National Natural Science Foundation (No. 81173017, No. 31101277), the National High-Tech Research and Development Program of China (863 Program) (No. 2014AA022303), Tianjin Science and Technology Program (No. 11ZCGHHZ01200, No. 12ZXCXSY08400, No. 12JCQNJC08900), and Tianjin Research Program of Application Foundation and Advanced Technology (13JCZDJC29700).

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