Journal of Pharmaceutical Analysis 8 (2018) 297–301

Contents lists available at ScienceDirect

Journal of Pharmaceutical Analysis

journal homepage: www.elsevier.com/locate/jpa
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Original Research Article

A rapid reporter assay for recombinant human brain natriuretic peptide (rhBNP) by GloSensor technology

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ARTICLE INFO

Article history:
Received 29 November 2017
Received in revised form
17 April 2018
Accepted 18 April 2018
Available online 20 April 2018

Keywords:
RhBNP
cGMP
GloSensor technology
Reporter assay

ABSTRACT

Accurate determination of biological activity is essential in quality control of recombinant human brain natriuretic peptide (rhBNP). In previous study, we successfully developed a genetically modified cell line 293GCAC3-based ELISA assay for rhBNP. But ELISA procedure is still tedious, so this study was aimed to develop a rapid and simple bioassay for rhBNP using GloSensor technology, which provides a platform of flexible luciferase-based biosensors for real-time detection of signaling events in live cells, including cGMP production. A reporter cell line 293GCAGlo-G1 was constructed by transfecting pGloSensor™ 40 F plasmid into 293GCAC3. The reporter assay based on 293GCAGlo-G1 showed high precision with intra-assay CV being 8.3% and inter-assay CV being 14.1%; high accuracy with 80%, 100% and 120% recovery rate being 99.2%, 102.4% and 99.0% respectively; and great linearity with R^2 of linear fitting equation being 0.99. Besides, no significant difference was found in test results of reporter assay and 293GCAC3-based ELISA assay (paired t test, $p = 0.630$). All these results suggested that the reporter assay was a viable assay for biological determination of rhBNP.

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1. Introduction

B-type natriuretic peptide (BNP), also called brain natriuretic peptide, is produced primarily by the ventricular myocardium in response to volume and pressure overload [1–3]. Recombinant human B-type natriuretic peptide (rhBNP) was approved by FDA in 2001 to treat severe congestive heart failure and recommended in the acute decompensated heart failure (ADHF) treatment guidelines by the European Society of Cardiology (ESC) in 2005 [4–6]. It also obtained a China national new drug certificate and production license in 2005. It can mediate natriuretic, diuretic and smooth muscle relaxant effects, and thus decrease in preload and afterload by venous and arterial vasodilation, which results in increased cardiac output [7–9]. A recent clinical study found that continuous administration of rhBNP can improve heart and renal function in patients after cardiopulmonary bypass surgery as well as accelerate the recovery from myocardial injury [10].

As rhBNP is used more and more widely in clinic, it is imperative to improve the quality standards of relevant pharmaceutical products. In previous study, we developed a 293GCAC3 cell-based ELISA assay to replace rabbit aortic strips test (RAST), which simplifies the experiment and improves accuracy and precision, and this cell-based assay has been widely applied within the industry in China [11]. But ELISA procedure is still very tedious, with repeated washing of plate, which surely would bring variation in measurements. Besides, since the concentration of different batches of commercial anti-cGMP antibodies and HRP-cGMP is not uniform, the dilution rates of working solutions need to be reconfirmed by pre-experiments when new batches of reagents are used. Here we introduce a rapid bioassay for rhBNP using GloSensor technology, which provides a platform of flexible luciferase-based biosensors for real-time detection of signaling events in live cells, including cAMP, cGMP and protease activity. pGloSensor™ cGMP (Promega) uses genetically encoded biosensor variant with cGMP binding domain fused to mutant form of Photinus pyralis luciferase [12,13]. Upon binding to cGMP, conformational changes occur, promoting large increases in light output. The magnitude of the luminescence increase is directly proportional to the amount of rhBNP. This GloSensor technology was developed ten years ago and primarily applied in research on cellular signaling mechanism in the past few years. This is the first time for this technology to be used in bioactivity determination of bio-drugs, and our study provides a

Peer review under responsibility of Xi'an Jiaotong University.
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https://doi.org/10.1016/j.jpha.2018.04.003
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new way to detect agonists of guanylate cyclase / adenylate cyclase receptors and G protein-coupled receptors.

In this study we developed a reporter cell line 293GCAGlo-G1 by transfecting the plasmid pGloSensor™ cGMP into 293GCAC3 cells, which could produce increased light output to rhBNP stimulation. A reporter assay based on 293GCAGlo-G1 was subsequently established and validated, as well as compared with 293GCAC3 cell-based ELISA assay.

2. Materials and methods

2.1. Materials

293GCAC3 cell line was constructed by National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China. DMEM-high glucose, fetal calf serum, 0.25% trypsin-EDTA and G418 were from Thermo Fisher Scientific (Waltham, MA, USA). GloSensor™ cAMP reagent and pGloSensor™ 40F plasmid were from Promega (Madison, Wisconsin, USA). X-transgene 9 transfection reagent was from Roche (Basel, Switzerland). Hygromycin B was from Amresco (Englewood, Colorado, USA). 3-Isobutyl-1-Methylxanthine (IBMX) was from Sigma-Aldrich (Stockholm, Sweden). rhBNP reference (500 units per vial) and rhBNP samples were supplied by manufacturers (China).

2.2. Cell culture, transfection and clone selection

2.2.1. Cell culture

293GCAC3 cells were grown in DMEM-high glucose supplemented with 10% fetal calf serum and 200 μg/mL G418. Cell passaging was achieved by detaching the cells in 0.25% trypsin-EDTA and splitting the cells every 3 days.

2.2.2. Transfection

293GCAC3 cells (approximately 80% confluence) in 6-well plate were transfected with the plasmid using X-transgene 9 transfection reagent according to the protocol. After cultured in growth media for 48 h, cells were collected for next test.

2.2.3. Clone selection

293GCAC3 cells transfected with pGloSensor™ 40F were cultured in growth media for 48 h. Then the growth media was replaced with selective media containing 200 μg/mL G418 and 100 μg/mL hygromycin B. After growth in selective media for 4 weeks, resistant clones were subcloned by limited dilution and screened for the induction of light output by treatment of cells with gradient concentrations of rhBNP. The clone exhibiting the highest responsiveness to rhBNP was further characterized.

2.3. 293GCAC3-based ELISA assay

293GCAC3 cells in DMEM without antibiotics and serum were seeded in 96-well costar plates (1.8×10^4/well in a total volume of 180 μL) and incubated at 37 °C in a CO_2 incubator for 16–18 h. RhBNP reference or samples were gradiently diluted by 4 times in PBS buffer containing 1 mM IBMX and 0.1% BSA, and 20 μL rhBNP serial dilutions were added to the cell plate, which was then incubated at 37 °C in a CO_2 incubator for 1.5–2 h. Protein G precoated microtiter plate was incubated with 100 μL cGMP antibodies for 1 h. 50 μL culture supernatant and 50 μL HRP-cGMP conjugate were mixed, and put into cGMP antibody coated plate, shaking at room temperature for 3 h. Then the mixtures were discarded, and the plate was washed 4 times. 100 μL TMD substrates were put into the plate, reacting at room temperature for 10 min, and terminated by 100 μL stop buffer. OD_{450} values were then determined by reading on a SPECTRAmax plate reader.

2.4. Reporter assay

293GCAGlo-G1 cells in analysis medium (IMDM supplemented with 10% fetal calf serum) were seeded in 96-well white plates (4×10^4/well in a total volume of 50 μL), and incubated at 37 °C in a CO_2 incubator for 16–18 h. 30 μL equilibration medium (3 mL analysis medium supplemented with 200 μL of GloSensor™ cAMP reagent stock solution) was put into the cell plate and incubated at room temperature for 2 h. rhBNP reference or samples was gradiently diluted 2 times in analysis medium, and 20 μL rhBNP serial dilutions were added to the cell plate, which was then incubated at room temperature for 30–60 min. Luminescence values were determined by reading on a SPECTRAmax plate reader at set intervals.

2.5. Statistical analysis

Analyses of the data consisted of statistical models used to calculate EC_{50} value as well as statistical techniques for method validation. In order to calculate the EC_{50} values, dose response and linear range, we used the 4-PL model. Analyses were carried out using GraphPad Prism 5 and SigmaPlot 12 for EC_{50} calculations and method validation.

3. Results

3.1. Responsiveness of 293GCAC3 transfected with pGloSensor™ 40F to rhBNP stimulation

As the initial step, we transiently transfected 293GCAC3 cells with the plasmid pGloSensor™ 40 F and tested whether it could produce increased light output to rhBNP stimulation. 5 h after transfection, equilibration medium was added and incubated at room temperature for 2 h, followed by rhBNP stimulation (serial concentrations) for 90 min and the light output was detected at set intervals. The results are shown in Fig. 1. The results indicated that pGloSensor™ 40F transfected 293GCAC3 cells produced increased levels of light output in response to the ascending concentrations of rhBNP, and the increase of light output was the highest between 30 and 40 min. As the cells transiently transfected with pGloSensor 40 F demonstrated excellent responsiveness to rhBNP treatment, we then set out to develop a stable reporter cell line.

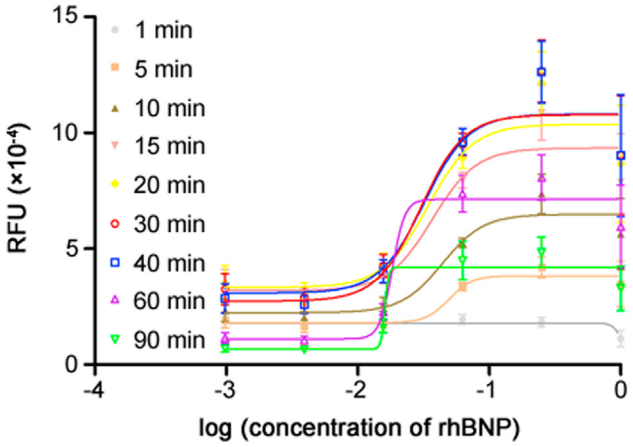

Fig. 1. Responsiveness of 293GCAC3 transfected with pGloSensor 40F to rhBNP stimulation. 293GCAC3 cells transfected with pGloSensor 40F were stimulated by rhBNP dilutions and the light output was determined at set intervals (5, 10, 15, 20, 25, 30, 40, 60 and 90 min). Each point and error bar represents the mean and standard deviation of three replicates, respectively.

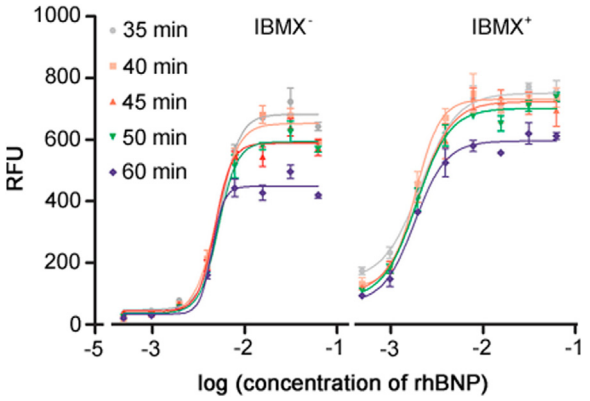

Fig. 2. The effect of IBMX on the reporter assay. 293GCAGlo-G1 cells were stimulated by rhBNP dilutions with or without the presence of IBMX(1 mM). Each point and error bar represents the mean and standard deviation of two replicates, respectively.

3.2. Development of a reporter assay for rhBNP

293GCAC3 cells were transfected with pGloSensor™ 40F, and cultured in selective media containing 100 μg/mL hygromycin B for 4 weeks. Positive clones were obtained by limited dilution. As shown in Fig. S1, clone G1 was found to produce highest level of light output in response to rhBNP treatment, which was next employed to develop a reporter assay for rhBNP. Various parameters of the assay were optimized, including cell number (40, 000 per well), concentration range of rhBNP (.002–0.25 μg/mL), and stimulation time (30–40 min). Given the presence of IBMX, an inhibitor of phosphodiesterase (PDE), is essential in ELISA assay, we tested the influence of IBMX (1 mM) in this reporter assay. As shown in Fig. 2, with the presence of IBMX, the magnitude and stability of light output improved slightly, but the background light output was significantly increased (about 3 times), which was against the accuracy and precision of the assay. So for the reporter assay, IBMX was unnecessary. The parameters of dose-response curves of 293GCAGlo-G1cells stimulated by rhBNP dilutions with or without the presence of IBMX are also listed in Table 1, including slope, minimum and maximum light outputs, EC_{50} and R^2. Although the light outputs changed over time, the EC_{50} values were relatively stable (CV was 7.7% for $IBMX^-$ and 5.8% for $IBMX^+$).

3.3. Precision, linearity, and accuracy

To validate the reporter assay, all tests were conducted according to ICH Guidelines, including precision, linearity, and accuracy. Five repeated analyses of rhBNP sample in one test or five different tests were conducted to evaluate intra- or inter-assay precision. Intra-assay CV was 8.3% and inter-assay CV was 14.1%, demonstrating high precision. Accuracy was evaluated by testing recovery rates, and the specific approach was that 80%, 100% and 120% rhBNP references were mixed with 100% rhBNP sample and tested simultaneously. The recovery rate was represented by the percentage rate of the difference between measured value of mixture and measured value of 100% rhBNP sample to expected value of rhBNP reference in mixture. The 80%, 100% and 120% recovery rates were 99.2%, 102.4% and 99.0% respectively, demonstrating great accuracy of the assay. Linearity was evaluated by testing 25%, 50%, 75%, 100% and 125% of rhBNP samples, and the linear fitting equation ($y = 0.99\,x - 0.01$, $R^2 = 0.99$) represents the correlation between them, where x is expected value and y is measured value. The slope of 0.99 suggested high correlations between expected values and measured values, and R^2 of 0.99 showed great linearity.

3.4. Comparison of reporter assay with ELISA assay

The agreement between reporter assay and ELISA assay was assessed by testing various samples using both methods. Three rhBNP bulks and three rhBNP products were tested by both methods, and the results are listed in Table 2. Paired *T* test showed no significant difference between two methods ($p = 0.630$), suggesting the consistency of two methods in test results. Then we compared two methods in materials, operation steps, sensitivity, signal to noise ratio (SNR) and precision (Table 3). The two methods were similar in sensitivity and precision, but the reporter assay was more rapid and simpler.

4. Discussion

Bioactivity determination is a critical quality attribute (CQA) for quality control of biological drugs, including rhBNP. The biological activity of this peptide drug was determined by RAST earlier, which is known to be laborious and time-consuming, with poor reproducibility and isolation of fresh aortic strip from sacrificed rabbit [14,15]. Numerous attempts have been made over decades to develop alternative assays aiming at reduced use of animals and improved precision and robustness. The biological action of BNP is mediated by its main receptor natriuretic peptide receptor-A (NPR-A)/ guanyl cyclase site (GC-A), which has a guanyl cyclase site. Activated GC-A receptor could catalyze the conversion of guanosine triphos-phate (GTP) to cyclic guanosine monophosphate (cGMP), a second messenger triggering potent physiological actions [16–19]. Given the well-characterized pathways

Table 1.
The parameters of dose-response curves of 293GCAGlo-G1 cells stimulated by rhBNP dilutions with or without the presence of IBMX.

IBMX	Parameter	35 min	40 min	45 min	50 min	60 min	Average	RSD (%)
$IBMX^-$	Hillslope	3.98	3.63	4.30	4.42	7.57	4.78	33.3
	Min light output	43.41	32.35	37.75	35.92	31.70	36.22	13.1
	Max light output	663.52	634.83	557.64	558.72	422.15	567.37	16.5
	EC_{50}(ng/mL)	5.38	5.09	4.76	5.02	4.37	4.92	7.7
	R^2	0.98	0.97	0.95	0.96	0.95	0.96	1.4
$IBMX^+$	Hillslope	2.44	3.09	2.27	2.75	2.62	2.63	11.8
	Min light output	150.22	106.59	88.75	87.83	65.31	99.74	31.9
	Max light output	724.20	716.13	709.02	666.20	575.60	678.23	9.1
	EC_{50}(ng/mL)	2.04	1.85	1.97	1.82	1.77	1.89	5.8
	R^2	0.93	0.95	0.95	0.95	0.96	0.95	0.9

Table 2.
Test results of rhBNP samples by ELISA assay and reporter assay.

Sample	ELISA assay (units/mL)	Reporter assay (units/mL)
Bulk 01	2588	2714
Bulk 02	2835	2666
Bulk 03	2040	2102
Product 01	341	382
Product 02	616	668
Product 03	514	528

Paired T test, $p = 0.630$.

activated by BNP, quantification of cGMP in cells exposed to rhBNP has been explored as attractive alternative assay. Indeed, several types of cGMP-involved assays for rhBNPs have been reported in recent years, including the measurement of cGMP in human umbilical vein endothelial cells (HUVEC) or PC12 cells by radioimmunoassay, as well as a modified cell line 293GCAC3 by ELISA assay, which was developed by our previous work [11].

In this study, a new reporter assay was explored based on GloSensor technology, a platform technology of biosensors for the intracellular detection of signal transduction in living cells developed by Promega Corporation [13,14,20]. The plasmid pGloSensor™ 40F encodes a biosensor variant with cGMP binding domain fused to a mutant form of Photinus pyralis luciferase. Upon binding to cGMP, conformational changes occur, promoting large increases in light output. A reporter cell line was constructed by transfecting pGloSensor™ 40F into GCA-overexpressing cell line 293GCAC3. Fig. 3 describes the principle of this new reporter assay. Method validation was conducted according to ICH Guidelines, including precision, linearity, and accuracy. The reporter assay showed high precision with intra-assay CV being 8.3% and inter-assay CV being 14.1%; high accuracy with 80%, 100% and 120% recovery rate being 99.2%, 102.4% and 99.0% respectively; and great linearity with R^2 of linear fitting equation being 0.99. Given the specificity of reporter assay was consistent with ELISA assay, which has been validated in former study [11], it is unnecessary to evaluate it again. Besides, no significant difference was found in results of reporter assay and 293GCAC3-based ELISA assay (paired T test, $p = 0.630$). The two methods were similar on sensitivity and precision, but the reporter assay is more rapid and simpler for avoidance of ELISA steps.

5. Conclusion

Collectively, all our results suggested that the reporter assay was a viable assay for biological determination of rhBNP products,

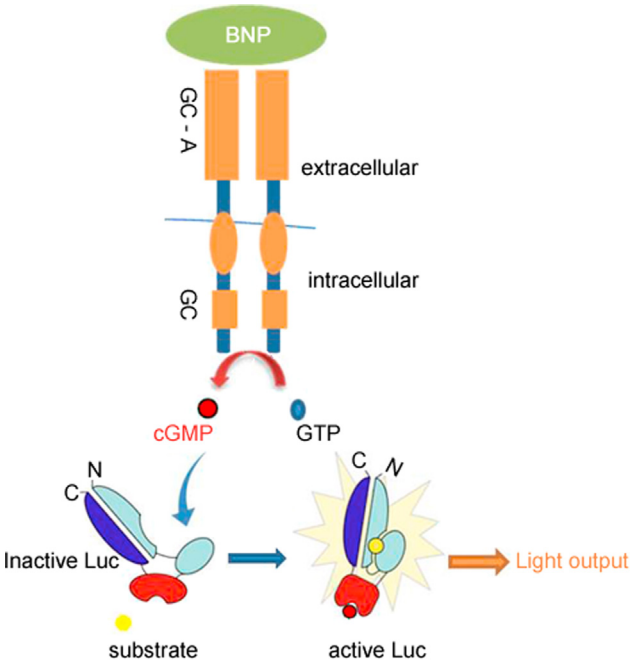

Fig. 3. A sketch for the principle of new reporter assay.

and single-reagent and one-step determination made the assay more controlled and suitable in routine inspection compared with former ELISA assay. What is more, our study provides a new way to detect agonists of guanylate cyclase/adenylate cyclase receptors and G protein-coupled receptors, which would promote the research and development (R&D) of these types of bio-drugs.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

This work was financially supported by grants from the National Science and Technology Major Project (No. 2015ZX09501008-001) and the Middle-aged and Young Development Research Foundation of NIFDC (No. 2017B3). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Table 3.
Comparison between ELISA assay and reporter assay.

Materials, method and result	ELISA assay	Reporter assay
Cell line	293GCAC3	293GCAGlo-G1
Extra materials	IBMX, HRP-cGMP, anti-cGMP antibody, dilution buffer, wash buffer, TMB substrate, stop solution, Protein G-coated plate	GloSensor™ cAMP Reagent, White cell plate
Experiment steps	1. Preparation of cell plate (16–18 h) 2. Preparation of BNP samples and stimulation for 1.5–2 h 3. Immobilization of anti-cGMP antibody (1 h) 4. Competitive ELISA test (3–4 h) 5. Determination	1. Preparation of cell plate (16–18 h) 2. Equilibration for 2 h 3. Preparation of BNP samples and stimulation for 0.5–1.5 h 4. Determination
Sensitivity(EC_{50})	~5 ng/mL	~5 ng/mL
SNR (signal to noise ratio)	> 2.5	> 10
Intra-assay CV	< 10%	< 10%
Inter-assay CV	< 20%	< 20%

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jpha.2018.04.003.

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