



Development, Optimization, and Validation of an *in vitro* Cell-Based Bioassay to Determine the Biological Activity of Teriparatide (PTH1–34)

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Abstract

This study aimed to establish an efficient *in vitro* cell-based assay to measure the activity of teriparatide (PTH1–34). In this study, a rat osteosarcoma cell line (UMR-106) was treated with various concentrations of PTH1–34, and the biological activity of PTH1–34 was determined by quantitatively measuring intracellular cyclic adenosine monophosphate levels using a time-resolved fluoroimmunoassay. A four-parameter fitting analysis was used to calculate the relative potency of the samples. The experimental conditions were optimized. The method's specificity, relative accuracy, precision, and linearity were validated. Our data suggested that this method had good specificity, a relative bias of relative accuracy ranging from –0.8 to 1.4%, a correlation coefficient for the linear regression equation of 0.9953, a geometric coefficient of variation for intermediate precision ranges from 2.0 to 3.5%, and a linear range of 50 to 150%. This method significantly improves the quality control and release inspection efficiency of PTH1–34 and may be further developed and validated as an alternative to the existing United States Pharmacopeia and European Pharmacopoeia inclusion methods. This method also provides a platform for the high-throughput screening of PTH1–34 analogs.

Keywords

- ▶ teriparatide
- ▶ *in vitro* bioassay
- ▶ cAMP
- ▶ time-resolved fluoroimmunoassay
- ▶ method verification

Introduction

Teriparatide (PTH1–34) was the first anabolic drug to be approved by the U.S. Food and Drug Administration (FDA) for bone reconstruction.^{1,2} Following the expiration of the patents on the original drug, many generics were extensively developed and have received approval worldwide. Bioactivity is a very important attribute of polypeptide drugs, including PTH1–34. Therefore, establishing a sensitive, efficient, and stable method to demonstrate the similarity of biosimilars

or generic drugs with pharmacopeia standards and reference medicinal products is required.

The current edition of the United States Pharmacopeia (USP, 2021) includes an *in vitro* cell-based biological activity assay to evaluate the potency of PTH1–34. According to this pharmacopeia, this method requires the seeding of samples 24 hours in advance, a 24-hour starvation period, and drug detection, which takes 2 to 3 hours, which is a long time. The required test reagents must also be ordered 1 year in advance, which is expensive and unsuitable for high-throughput testing. Because

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of the high cost of this testing method, challenges in sourcing the reagents, and the significant time and cost required for testing, there is an urgent need to improve the *in vitro* activity testing methodology.

In this study, a novel cell-based *in vitro* assay was established to measure the activity of PTH1–34. The N-terminus of PTH1–34 binds to the near-membrane structure of PTH1R and activates G protein signaling.^{3–5} When UMR-106 cells are coincubated with PTH1–34, cyclic adenosine monophosphate (cAMP) signals are generated through the G protein-coupled receptor signaling pathway.^{5,6} In the present study, UMR-106 cells were coincubated with PTH1–34, and a time-resolved fluoroimmunoassay was used to quantify intracellular cAMP levels to assess the activity of PTH1–34.⁷ The principle is based on the fact that the labeled cAMP tracer competes with the PTH1–34-induced cAMP in cells through binding to labeled cAMP antibodies. PTH1–34-induced cAMP levels can be determined by measuring the concentration of the labeled cAMP when the labeled antibody binds to the labeled cAMP. We comprehensively validated this method and demonstrated its feasibility as a high-throughput assay for PTH1–34 biological activity.

Materials and Methods

Instruments and Reagents

The instruments used in this study were an electric constant temperature sink (model HH-420, Changzhou Zhiborui Instrument Manufacturing Co., Ltd., Changzhou, China), ultrapure water meter (model Millipore/Milli-Q Advantage A10, Merck, Germany), CO₂ incubator (model CCL-1708-8, Esco, Singapore), inverted microscope (model CKX53, Olympus, Japan), type A2 biosafety cabinet (model AC2-4S1, Esco, Singapore), benchtop centrifuge (model TD6, Lu Xiangyi, Shanghai, China), microplate thermostatic oscillator (model BE-9008, Haimen Qilinbell Instrument, HaiMen, China), multifunctional microplate reader (model SpectraMax i3X, MD, United States), and electronic balance (model SQP, Sartorius, Germany).

The materials used in this study were Dulbecco's Modified Eagle Medium (DMEM) (lot number 12800-017, Gibco, United States), penicillin-streptomycin (lot number 15140-122, Gibco, United States), bovine albumin (batch number 69003435, Sino-pharm, Shanghai, China), fetal bovine serum/FBS (batch number 10099-141, Gibco, United States), trypan blue (lot number 2101673, Gibco, United States), LANCE Ultra cAMP Kit (lot number TRF0263, PerkinElmer, United States), white 384-well plate (lot number 6008280, PerkinElmer, United States), and recombinant teriparatide (lot number 410019-201801, National Institutes for Food and Drug Control, China). PTH1–34 API, PTH1–34 preparation, PTH1–30 impurity, and adrenocorticotropic hormone (ACTH) were purchased from Shanghai Duomirui Biotechnology Ltd., (Shanghai, China). The UMR-106 cell line was obtained from the Chinese Academy of Sciences Cell Bank (lot number TCR11, Shanghai, China).

Reagent Preparation

The analysis media were prepared under sterile conditions. DMEM basal medium (99 mL, containing 3.7 g/L sodium bicarbonate) was supplemented with bovine serum albumin

(0.1 g) and penicillin-streptomycin (1 mL). The PTH1–34 reference and samples were diluted to specific concentrations using this media.

Bioactivity Assay

UMR-106 cells were cultured in DMEM medium with 10% FBS and 1% penicillin-streptomycin in the presence of 3.7 g/L sodium bicarbonate at 37°C in a 5% CO₂ atmosphere.

Viable cells were stained with trypan blue, and counted under an inverted microscope. A total of 10³ cells/well in 5 µL of analysis medium were added to each well of a 384-well plate. The PTH1–34 reference and samples were at an initial concentration of 4,000 ng/mL and 3-fold serial dilutions were prepared in the analysis medium. Then, 5 µL of serially diluted PTH1–34 reference and the samples were added to each well. After a 30-minute incubation period at 25°C in the dark and the addition of 10 µL of LANCE Ultra cAMP reagent, the samples were incubated for 60 minutes at 25°C in the dark. The 384-well plate was placed on an MD microplate reader for TR-FRET detection and the detection procedure is shown in ►Table 1.

Method Optimization

Dilution Rate

Using PTH1–34 API at 4,000 ng/mL as the initial concentration, 2-fold (1:1), 3-fold (1:2), and 5-fold (1:4) gradient dilutions were prepared (9 gradients each) and the potency levels of PTH1–34 was detected according to “Bioactivity Assay.”

Cell Seeding Density

Using PTH1–34 API at 4,000 ng/mL as the initial concentration and a 3-fold dilution, the cell density was adjusted to obtain 250, 500, 1,000, and 2,000 cells/well and the potency levels of PTH1–34 was detected according to “Bioactivity Assay.”

Method Verification

Specificity

Recombinant teriparatide, PTH1–34 API, PTH1–34 preparation, PTH1–30 impurity, ACTH, and the prepared blank

Table 1 SpectraMax i3X program settings

Detect items	Set parameters
Cartridges, read modes, read type	HTRF, TR-FRET, endpoint
Wavelengths	Excitation: 340 nm Emission: 615/665 nm
PMT and optics	Integration time: 0.2 ms Excitation time: 0.05 ms Number of pulses: 30 Measurement delay: 0.02 ms Read from top Read height: 8.25 mm
Shake	No shake
Read order	Row
Show optimizer	On

Abbreviation: HTRF, homogeneous time-resolved fluorescence; TR-FRET, time-resolved fluorescence resonance energy transfer.

excipient were diluted to 4,000 ng/mL as an initial concentration, and a 3-fold gradient dilution was established and the potency levels of PTH1–34 was detected according to “Bioactivity Assay.”

Relative Accuracy, Intermediate Precision, Linearity, and Range

PTH1–34 API (10 mg) was diluted to 10 mg/mL with analysis medium (a total of six parts were diluted, one of which was used as a standard solution and the other five as a test solution), and diluted to 4,000 ng/mL as the standard solution in analysis medium and the mass concentration of 2,000, 2,800, 4,000, 5,200, and 6,000 ng/mL were established as test solutions. The relative potency levels of five analyte solutions were 50, 70, 100, 130, and 150%, respectively, and the activity of PTH1–34 was detected according to “Bioactivity Assay.” Each potency level was assessed by two analysts using three cell generations on three different dates, and the data are presented as measured potency. Measured potency = $R/X \times 100\%$, where R is the EC_{50} value of the standard solution and X is the EC_{50} value of the test solution.

Robustness

UMR-106 cells were cultured in DMEM medium containing 10% FBS and 1% penicillin–streptomycin in the presence of 3.7 g/L sodium bicarbonate at 37°C in a 5% CO₂ atmosphere and passaged 1:1 every other day. The cells were passaged to the 4th, 11th, and 25th generations, and the effect of each cell generation on the 100% potency level was determined according to the “Bioactivity Assay.”

Data Analysis and Statistics

All statistical analyses were performed using SoftMaxPro version 7.1.2 and GraphPad Prism 9.5 software. Taking the sample concentration as the x-axis and the corresponding fluorescence ratio as the y-axis, a four-parameter equation was selected for fitting, and the dose–response curves of the active standard and the test product were generated to obtain the concentration for 50% of the maximal effect (EC_{50}) value, which was calculated by the following formula: fluorescence ratio = $EM_{665\text{ nm}}/EM_{615\text{ nm}}$; EM, emission.

Results

Dilution Ratio

The bioassay conditions were optimized by evaluating two parameters: dilution ratio and cell density. The first optimized parameter was the dilution ratio, where the fluorescence ratios were measured for different concentrations of PTH1–34 API with a dilution ratio of 1:1, 1:2, and 1:4, respectively. As shown in ►Fig. 1, the data point distribution of the four-parameter curve obtained under the 1:1 dilution ratio was not good and not considered, and the data point distribution of the four-parameter curve obtained under the 1:2 dilution ratio was more uniform than that obtained at 1:4 dilution ratio. The logarithmic period data points were greater than those of the 1:4 dilution ratio, which can truly reflect the data fitting situation. The calculated results were

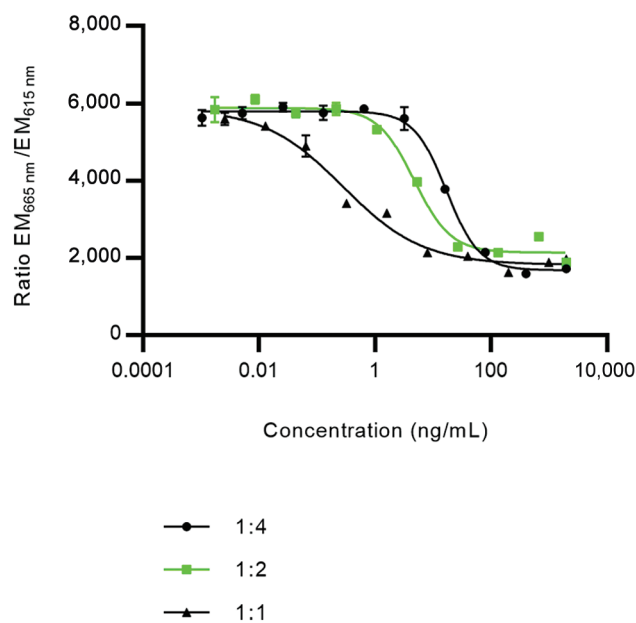


Fig. 1 Optimization of the dilution ratio ($n = 3$).

more reliable; thus, the optimal dilution ratio for this method was 1:2.

Cell Density

The second optimized parameter was cell density, where the fluorescence ratios were measured for different concentrations of PTH1–34 API using cell densities of 250, 500, 1,000, and 2,000 cells/well, respectively. As shown in ►Fig. 2, the four cell densities yielded a typical S-type curve, and the R^2 values were 0.867, 0.945, 0.984, and 0.979,

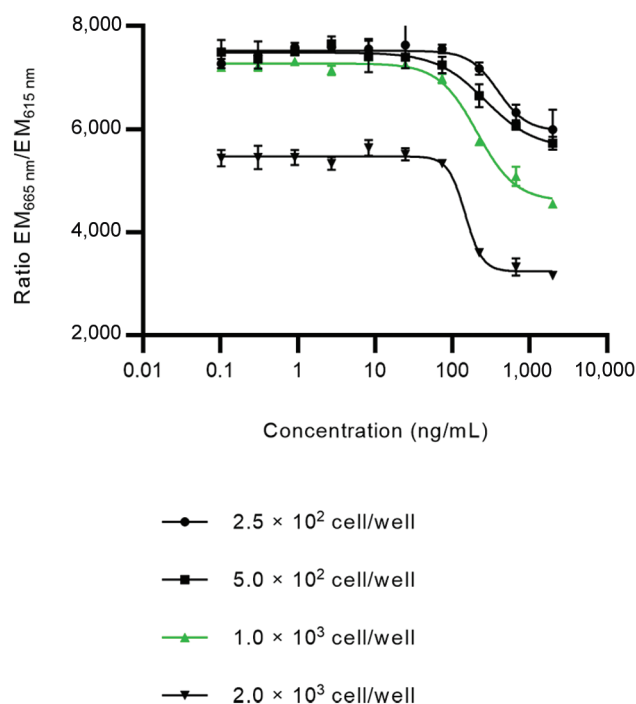


Fig. 2 Optimization of the cell density ($n = 3$).

respectively. The window of the four-parameter fitting curve for 250 and 500 cells/well was very narrow. The R^2 values for 1,000 and 2,000 cells/well were very close, and the window range of the four-parameter fitting curve was close; however, the 1,000 cells/well density dropped more in the inflection point value of the four-parameter fitting curve. Thus, a cell density of 1,000 cells/well was considered optimal.

Method Verification

The optimized conditions were applied for further validation according to the 2020 edition of the Chinese Pharmacopoeia (ChP). The validation characteristics are described below.

Specificity

Specificity refers to the ability of an assay to correctly measure an analyte without interference from other components, such as impurities, degradation products, and matrices. We selected recombinant teriparatide, PTH1–34 API, PTH1–34 preparations, PTH1–30 impurities, ACTH, preparation blank excipients, and drug dilution solution (analysis medium) for the treatment of cells to study the specificity of the method. As shown in ►Fig. 3, the same concentration of PTH1–30 impurity, ACTH, PTH1–34 preparation control (blank excipients), and drug dilution solution could not stimulate the production of cAMP in UMR-106 cells; however, the same concentration of recombinant teriparatide, PTH1–34 API, and PTH1–34 preparation stimulated the production of cAMP in UMR-106 cells. They yielded a good dose–response relationship, indicating that the biological activity detection method of PTH1–34 was unique.

Relative Accuracy, Intermediate Precision, Linearity, and Range

The method was evaluated for relative accuracy, intermediate precision, linearity, and range, and the four indices were verified as a combined design. The potency was measured using five levels (50, 70, 100, 130, and 150%) of prediluted initial working concentrations of PTH1–34 API (i.e., 4,000 ng/mL). Each potency level was determined by two analysts using three cell generations on three different days. The results of six different assays are shown in ►Table 2. The results of the following four validation indexes are based on the results of these six measurements.

Table 2 Results for six different assays

Expected potency (%)	Measured potency					
	Analyst 1			Analyst 2		
	Day 1 (%)	Day 2 (%)	Day 3 (%)	Day 1 (%)	Day 2 (%)	Day 3 (%)
150	148.1	147.2	149.2	152.0	148.2	152.6
130	133.2	126.4	125.5	133.1	130.9	132.0
100	98.1	99.8	102.3	99.7	102.2	97.8
70	67.3	70.5	73.5	70.2	66.7	68.2
50	47.7	50.7	55.8	52.2	50.6	47.2

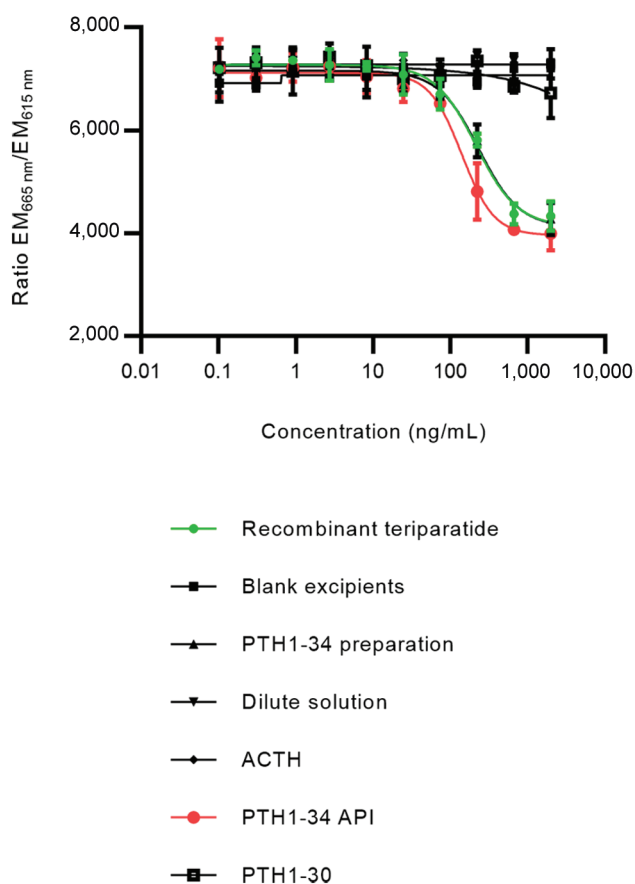


Fig. 3 Specificity curve ($n = 2$).

Linearity and Range

To obtain a linear relationship between the measured relative valence and the true value or reference value within the design range, a linear regression equation was generated from the results of the six biological activities measured in ►Table 2. As shown in ►Fig. 4, the regression equation ($y = 0.9951x + 0.4542$, $R^2 = 0.9953$) exhibited a linear correlation.

Relative Accuracy

Relative accuracy refers to the degree to which the measured relative potency is close to the true value or reference value within a specified range, generally using relative bias (RB, %)

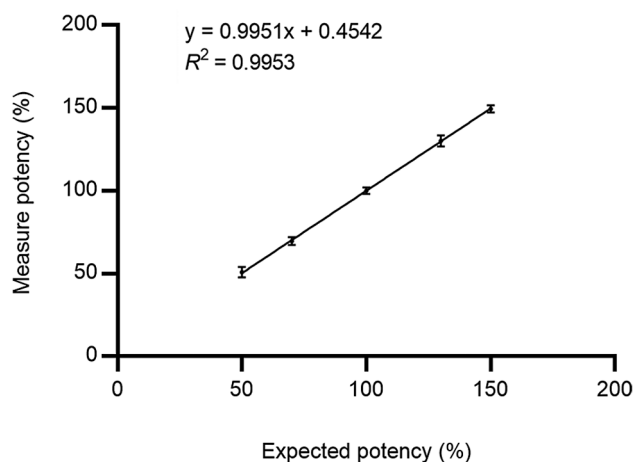


Fig. 4 Linearity and range ($n = 6$).

for assessment. In this work, the RBs and confidence intervals (CIs) at each potency level were calculated according to the ChP 2020 General Rule 9401 (►Table 3). The results showed that the average RB of the relative potency measurements at the five potency levels ranged from -0.8 to 1.4% , and the RB was within the range of $\pm 12\%$, which complies with the pharmacopeia standard.

Intermediate Precision

Intermediate precision refers to the proximity between the results of the same homogeneous test sample measured by multiple samples under the specified conditions. Based on the ChP 2020 General Rule 9401, the geometric standard deviation and geometric coefficient of variation (GCV) of the five relative potencies were measured for each potency level. The intermediate precision was evaluated using the GCV for each potency level. The results are listed in ►Table 4. Our data showed that the GCV range was 2.0 to 3.5% and the GCV at the five potency levels was less than 25%. The relative accuracy, intermediate precision, linearity, and range verification results of the five potency levels indicated that the test

Table 4 Geometric standard deviation and geometric coefficient of variation of relative potency measured at different potency levels

Potency level (%)	Number of trials	GSD	GCV (%)
150	6	1.0227	2.3
130	6	1.0347	3.5
100	6	1.0197	2.0
70	6	1.0254	2.5
50	6	1.0321	3.2

Abbreviations: GCV, geometric coefficient of variation; GSD, geometric standard deviation.

Note: $GSD = \text{antilog}(SD)$, where SD is the logarithmic standard deviation of the potency determination at each potency level; $GCV = (GSD - 1) \times 100\%$.

results of this method meet the requirements of General Rule 9401 in the range of 50 to 150%. Thus, the relative potency of the method was 50 to 150%.

Robustness

As shown in ►Fig. 5 and ►Table 5, the S-type dose–response curves could be obtained for all three generations of cells, with actual potency measurements of 114.2, 97.3, and 77.5%, respectively. Fluctuations in the range of 70 to 130% indicated that they were not affected and that the potency values of the three generations were very close. Given the above, the cell generation at the 4th to 25th passage had no significant effect on the measured biological activity results.

Discussion

PTH1–34 is an anabolic (bone-forming) agent that is effective in postmenopausal women with osteoporosis and patients with glucocorticoid-induced osteoporosis.⁸ Abaloparatide, engineered from PTHrP (1–34), was approved by the FDA in April 2017 for the treatment of osteoporosis in

Table 3 Relative bias and confidence intervals for relative potency measures at different potency levels

Theoretical potency level (%)	Number of trials	Logarithmic potency			Measured potency			RB		
		Average value	LCI	UCI	Average value (%)	LCI (%)	UCI (%)	Average value (%)	LCI (%)	UCI (%)
150	6	0.402	0.390	0.415	149.6	147.7	151.4	−0.3	−1.5	0.9
130	6	0.263	0.242	0.285	130.2	127.4	133.0	0.1	−2.0	2.3
100	6	0	−0.016	0.016	100.0	98.4	101.6	0	−1.6	1.6
70	6	−0.366	−0.395	−0.336	69.4	67.3	71.5	−0.8	−3.8	2.1
50	6	−0.681	−0.732	−0.630	50.7	48.1	53.3	1.4	−3.8	6.5

Abbreviations: CI, confidence interval; LCI, lower confidence limit; RB, relative bias; SD, standard deviation; UCI, upper confidence limit.

Notes: Logarithmic potency = $\ln(\text{measured potency})$, the base for logarithmic conversion in the table is e .

Relative bias = $(\text{measured potency}/\text{theoretical potency level} - 1) \times 100\%$.

CI = $\text{average} \pm t_{df} \times SD/n^{1/2}$; LCI = $\text{average} - t_{df} \times SD/n^{1/2}$; UCI = $\text{average} + t_{df} \times SD/n^{1/2}$, where the average is the logarithmic mean of the potency values for each potency level, SD is the logarithmic standard deviation of the potency determination at each potency level, n is the number of potency measurements for each potency level, t_{df} is the value of the t -test table when the degree of freedom is df , and df is the degree of freedom and equal to the number of measured values for each potency level minus 1.

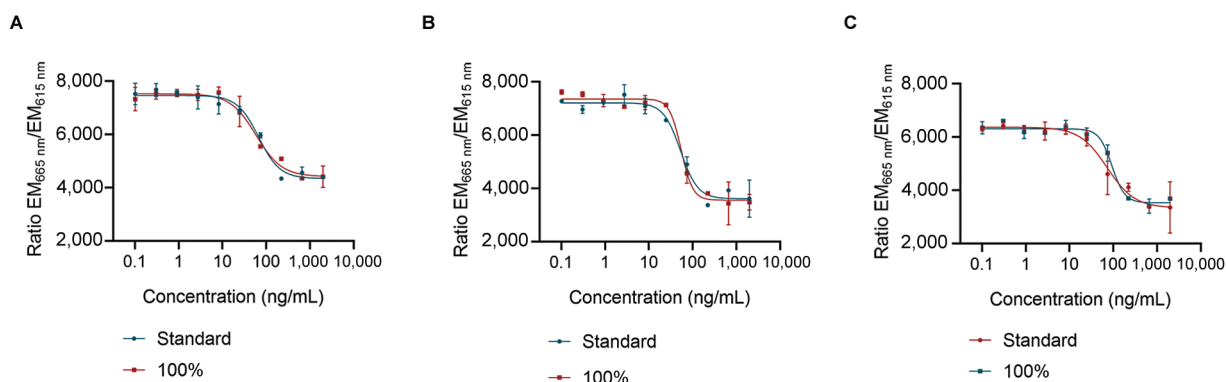


Fig. 5 Cell generation robustness ($n = 2$). (A) 4th generation. (B) 11th generation. (C) 25th generation.

Table 5 100% potency results for different cell generations

Generation	Theoretical potency (%)	Actual potency (%)	Average potency (%)
4 th	100	114.2	96.3
11 th		97.3	
25 th		77.5	

postmenopausal women.⁹ Abaloparatide binds to PTH1R. As a prominent new drug, PTH1R-related targets have again captured the interest of researchers developing potent and safer drugs to promote bone health.¹⁰ Although physical and chemical analysis of macromolecular drugs have become more advanced over time, even if they are detailed, they cannot always be determined from their high-order structures, they need to be inferred from the biological activity of the product. This indicates the importance of biological activity analyses. Regulatory agencies in various countries expect biological activity analyses to be based on the mechanism of action of the drugs; thus, cell-based biological activity methods are widely used for the release and stability analysis of macromolecule drugs.¹¹

In the USP rhPTH1–34 biological activity detection method, cells should be pretreated for approximately 48 hours, plated 24 hours in advance, and starved for 24 hours. This lengthy precell treatment may affect the cell state and the working solutions, which must be transferred twice. This undoubtedly increases experimental error and reduces the accuracy of the resulting data.¹² In the present study, UMR-106 cells directly interacted with rhPTH1–34 for 30 minutes in a one-step method, which greatly reduced cell preparation time and improved the efficiency of the assay.

Reporter gene methods have been widely used for cell viability assays in recent years.¹³ Reporter gene assays, which are the mechanism of action related, less variable, accurate, precise, and time-saving, are becoming increasingly recognized and adopted for quality control.¹⁴ Therefore, plasmids containing luciferase and overexpressing the PTHR1 gene may be used to transfer HEK293 and other cells to reduce the experimental period and improve the stability of methods through a reaction with luciferin substrates. The

method described here will accelerate the progress of current drug development.

Conclusion

In this study, drug dilution gradient and cell density were optimized. Moreover, the methodology was verified according to the USP and the Pharmacopeia of the People's Republic of China biological activity guidelines. The results indicate that the assay exhibits good precision, accuracy, specificity, and good linear relationship in a range of 50 to 150%. The results of passage stability studies show that the cells exhibited a good dose–response curve when passed up to 25 generations.

Conflict of Interest

None declared.

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