Tropical Journal of Pharmaceutical Research April 2015; 14 (4): 671-677
ISSN: 1596-5996 (print); 1596-9827 (electronic)
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Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v14i4.16

Original Research Article

Optimization, Validation and Application of Spectrophotometric Assay for 3-Hydroxy-3-methylglutaryl-coenzyme A Reductase Activity

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Received: 9 January 2015 *Revised accepted: 24 March 2015*

Abstract

Purpose: *To improve the sensitivity and specificity of spectrophotometric 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase activity assay.*
Methods: *Spectrophotometric HMG-CoA reductase detection in male Wistar rat liver microsomes was optimized by applying different conditions, such as reaction buffer pH, NADPH and protein concentration, and preincubation and reaction times. The optimal set of conditions was validated using HMG-CoA reductase inhibitors, namely, pravastatin, fluvastatin, and rosuvastatin. IC_{50} was calculated and compared with that of a radiochemical assay. Ginkgo biloba extract's (GBE50) inhibitory effect on HMG-CoA reductase activity was evaluated using the optimized spectrophotometric protocol.*
Results: *The optimum assay conditions were as follows: reaction buffer pH 7.0, 100 µM NADPH, 50 µM HMG-CoA, and 200 µg/mL microsomal protein. The preincubation and reaction times were 20 and 60 min, respectively, at 37 ºC. The IC_{50} of pravastatin, fluvastatin, and rosuvastatin under the optimum condition was 0.026, 0.015, and 0.007 µM while for radioisotope assay, it was 0.034, 0.049 and 0.0119 µM, respectively. GBE50 significantly inhibited HMG-CoA reductase activity in a concentration-dependent manner ($p < 0.05$).*
Conclusion: *These results suggest that HMG-CoA reductase activity can be detected using the improved spectrophotometric assay. This assay can facilitate the discovery and development of new HMG-CoA reductase inhibitors in vitro.*

Keywords: *Spectrophotometry, 3-Hydroxy-3-methylglutaryl-coenzyme A reductase activity, Cholesterol metabolism, Ginkgo biloba, Pravastatin, Fluvastatin, Rosuvastatin*

Tropical Journal of Pharmaceutical Research is indexed by Science Citation Index (SciSearch), Scopus, International Pharmaceutical Abstract, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

Mevalonic acid (MVA) is an important biochemical intermediate in cholesterol synthesis. High circulating cholesterol levels may induce atherosclerosis, which contributes to the occurrence of cardiovascular and cerebrovascular disorders and diseases, such as hypertension, coronary heart disease, thrombosis, and cerebral hemorrhage [1].

3-Hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase is the rate-limiting enzyme in the MVA to cholesterol biotransformation pathway. Accordingly, it plays a significant role in cholesterol homeostasis [2]. Although methods to determine HMG-CoA reductase mRNA and

protein expression in various regulatory mechanisms are well established [3], a sensitive, economical HMG-CoA reductase activity assay has not been developed. HMG-CoA reductase activity is conventionally assayed using elaborate radiochemical techniques [4], chromatographic techniques coupled with mass spectrometry (LC/MS) [5], or spectrophotometrically monitoring the decrease in the cofactor NADPH absorbance at 340 nm [6].

The radiolabeled assay is limited for several reasons, including the use of radioactive substances, the complicated procedure, the potential for radioactive contamination, and the high cost. LC/MS requires advanced and expensive instruments, which limits its use as a routine laboratory technique. Compared with these methods, spectrophotometry is rapid and simple. There is no need to separate the products or handle radioactive materials. Moreover, spectrophotometers are standard laboratory equipment, making them easily accessible. However, spectrophotometric assays do have some disadvantages. They are subject to high background NADPH hydrolysis if the protein sample comes from whole cells or purified organelles, and protein purification is complicated and expensive. If an optimized spectrophotometric assay that is both sensitive and simple were developed, it would be of great importance in basic and pharmacological research on cholesterol metabolism.

HMG-CoA reductase inhibitors effectively reduce cholesterol biosynthesis, which makes them valuable hypolipidemic agents and the drugs of choice for atherosclerosis. Thus, a large number of studies investigating HMG-CoA reductase inhibitors are underway to identify candidate drugs for treating hypercholesterolemic disorders [7].

The objective of this study was to develop an improved spectrophotometric HMG-CoA reductase activity assay by optimizing key factors in the reaction condition. Furthermore, we evaluated the HMG-CoA reductase inhibitory effects of Ginkgo biloba extract (GBE50), which has been implicated in cholesterol metabolism modulation [8,9], using the improved spectrophotometric technique.

EXPERIMENTAL

Chemicals and materials

Colestyramine was purchased from Life-care Pharmaceutical Co. (Nanjing, China), and NADPH was from Beyotime Biotechnology Co. (Shanghai, China). HMG-CoA, Pravastatin, Fluvastatin and Rosuvastain were purchased from Sigma-Aldrich (St. Louis, MO, USA). [14C]-HMG-CoA and [14C]-Mevalonolactone (2.14 GBq/mmoL) were obtained from GE Healthcare Life Sciences (Buckinghamshire, UK). GBE50 was purchased from XingLing Pharmaceutical Co. (Shanghai, China). All other chemicals and reagents were analytical grade.

Animals

The animal studies were approved by Ethics Committee of Wuhan University, and the study protocol was in accordance with the guidelines for the Care and Use of Laboratory Animals of the Chinese Animal Welfare Committee. Male Wistar rats (220 - 250 g) were obtained from the Experimental Center of Hubei Medical Scientific Academy (China, no. 2006-0005). The animals were housed in a light-controlled room with the dark period from 7:00 AM to 7:00 PM, and fed *ad libitum* with 2 % cholestyramine-supplemented powdered diet for 6 days to achieve maximal liver HMG-CoA reductase activity [10]. The animals were anesthetized using isoflurane and sacrificed by decapitation at 10:00 AM on the sixth day, and the liver were isolated, weighed, and immediately stored at -80 °C until subsequent analysis.

Liver microsome preparation

Rats liver microsomes were prepared by homogenization and differential centrifugation using established protocols [11]. In brief, the tissues were homogenized in three-fold volume ice-cold homogenization buffer A (100 mM sucrose, 50 mM KCl, 40 mM potassium phosphate, 30 mM potassium EDTA, pH 7.2) with a motor-driven, glass-Teflon Potter-Elvehjem homogenizer (JingKe Chemical Co., Shanghai). The homogenate was centrifuged for 15 min at 12,000 × g, the supernatant was removed and centrifuged again for 15 min at 12,000 × g, and the resultant supernatant was centrifuged for 60 min at 100,000 × g. The pellets were resuspended in buffer A and centrifuged at 100,000 × g for 60 min. Following that, the pellets were resuspended in buffer B (buffer A plus 10 mM dithiothreitol) using a hand-driven, all-glass Potter-Elvehjem homogenizer to prepare microsomal suspension. All of the operations were performed at 4 °C. The final suspension was stored at -20 °C.

HMG-CoA reductase solubilization

The Beg ZH method was used to solubilize the enzyme [11]. The microsomal suspension was

thawed at 37 °C water and an equal volume of buffer B supplemented with 50 % glycerol and preheated to 37 °C was added. The mixture was homogenized thoroughly with a hand-driven, all glass Potter-Elvehjem homogenizer and incubated at 37 °C for 60 min. The suspension was diluted three-fold with buffer B, homogenized again, and then centrifuged at 100,000 × g for 60 min at 25 °C. The supernatant containing solubilized HMG-CoA reductase was used for the activity assay. The supernatant protein concentration was determined using the Bradford method [12], and the protein concentration was adjusted to 1 mg/mL.

Spectrophotometric HMG-CoA reductase activity assay

The HMG-CoA reductase activity was determined spectrophotometrically using previously published protocols [7,13] with some modifications. Briefly, the total reaction volume was 1 mL. The reaction was preincubated at 37 ºC. Both the microsomal protein (total protein concentration 200 µg/mL) and 100 µM NADPH were added to buffer C (0.2 M KCl, 0.16 M potassium phosphate, 0.004 M EDTA, and 0.01 M dithiothreitol). The reaction was initiated with 50 µM HMG-CoA and was allowed to proceed for 60 min. When the reaction finished, the optical density at 340nm was measured using a UV-1601 spectrophotometer (Shimadzu, Japan). One unit of enzyme activity was defined as the amount of NADPH consumed by 1 mg enzyme per minute. The system was validated via inhibition studies using three HMG-CoA reductase inhibitors: pravastatin, fluvastatin and rosuvastatin.

Radiochemical HMG-CoA reductase activity assay

To validate the optimized spectrophotometric assay, an adaptation of the radiochemical method described previously [14] was used to compare HMG-CoA reductase activity inhibition by pravastatin, fluvastatin and rosuvastatin (positive controls). A 120-µL reaction volume consisted of the microsomal protein and 500 nmoL of NADPH (dissolved in the reaction buffer containing 0.1 M of triethanolamine and 10 mM of EDTA). The reaction was preincubated at 37 ºC for 5 min. Then the drug inhibition was evaluated using different concentrations: pravastatin (1.3, 12.9, and 129 µg/L), fluvastatin (0.7, 7.7, and 77.4 µg/L) and rosuvastatin (0.3, 3.2, and 32.3 µg/L).

The reaction was initiated with 5.17 nmoL of [14C]-HMG-CoA. The reaction proceeded for 15 min at 37 ºC and terminated by the addition of 30 µL of 6 M HCl. The resultant mixture was incubated at 37 ºC for another 15 min to allow the MVA to convert to mevalonolactone. The incubation mixture was centrifuged at 10,000 g for 5 min, and the supernatant was directly spotted on a Silica Gel 60 F254 TLC plate (Merck, Germany). The plate was developed in benzene:acetone (1:1, v/v).

Finally, the Rf 0.5-0.9 region was removed by scraping using a clean razor blade, and the [14C] radioactivity determined using a liquid scintillation counter. [14C]-mevalonolactone was used as a standard. The 50 % inhibitory concentration (IC_{50}) of the three drugs was calculated using the Statistical Package for Social Sciences (SPSS 17.5) software package.

Inhibitory effect of GBE50 on HMG-CoA reductase activity

GBE50's inhibitory effect in different concentrations (32.26, 322.6, and 3226 µg/L) on HMG-CoA reductase activity was tested using the optimized spectrophotometric assay.

Statistical analysis

The data were expressed as mean ± SD. SPSS 17.5 software was used for data analysis. Analysis of variance (one-way ANOVA) was used to compare the means of different groups. Statistical significance was defined as $p < 0.05$.

RESULTS

Reaction buffer pH and preincubation and reaction time duration

The pH of the spectrophotometric reaction buffer, buffer C, was adjusted to five different values: 6.0, 6.5, 7.0, 7.5, and 8.0 and the activity was assayed. The activity increased initially but decreased when the reaction buffer pH value was greater than 7.0. Accordingly, the optimal reaction buffer pH for maximum reductase activity was 7.0 (Fig 1A, $p < 0.05$ vs. pH 6.5).

The reaction mixtures with 200 µg microsomal protein were preincubated with 100 µM NADPH at 37 ºC for 0 – 40 min prior to the addition of 50 µM HMG-CoA (Fig. 1B). The reaction proceeded for 40 min at 37 ºC. HMG-CoA reductase activity slightly increased with increased preincubation time then decreased. Peak activity occurred at a preincubation time of approximately 20 min ($p < 0.05$ vs. 0 min).

The reductase activity increased linearly with increased reaction time until 60 min and then plateaued. Therefore, the optimal reaction time was 60 min (Fig 1C, $p < 0.05$ vs. 50 min).

NADPH and microsomal protein concentration

There was an approximately linear increase in reductase activity with increased microsomal protein concentrations, and a microsomal protein concentration of 200 µg/mL was an appropriate concentration to obtain higher HMG-CoA reductase activity (Fig 2B, $p < 0.05$ vs. 50 µg/L).

Validation and application of the optimized assay

Assay of three known HMG-CoA reductase inhibitors

All the three drugs, pravastatin, fluvastatin and rosuvastatin, inhibited HMG-CoA reductase activity in a concentration-dependent manner, and the linear correlations (r values) were 0.999 (Fig 3A), 0.98 (Fig 3B) and 0.87 (Fig 3C), respectively. The IC_{50} of pravastatin, fluvastatin and rosuvastatin were 0.026, 0.015 and 0.0066 µM, respectively.

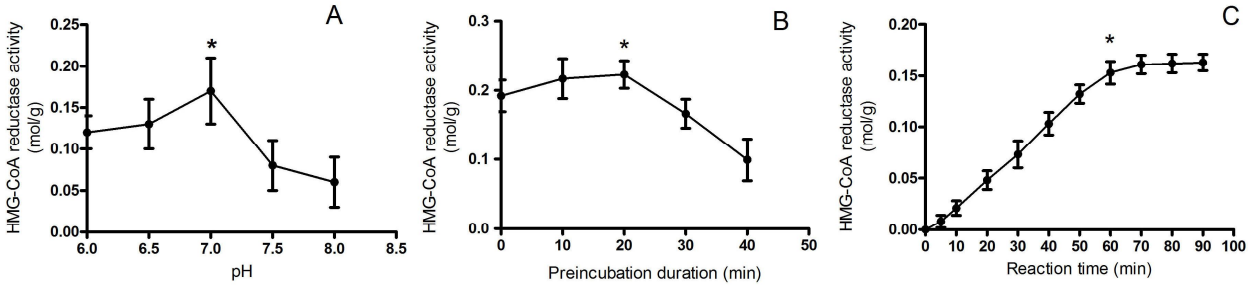

Figure 1: Effects of different reaction buffer pH (A), preincubation duration (B) and reaction time (C) on 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase activity. Data are presented as mean ± SD, n=5; $^{*}p < 0.05$ *vs* control group

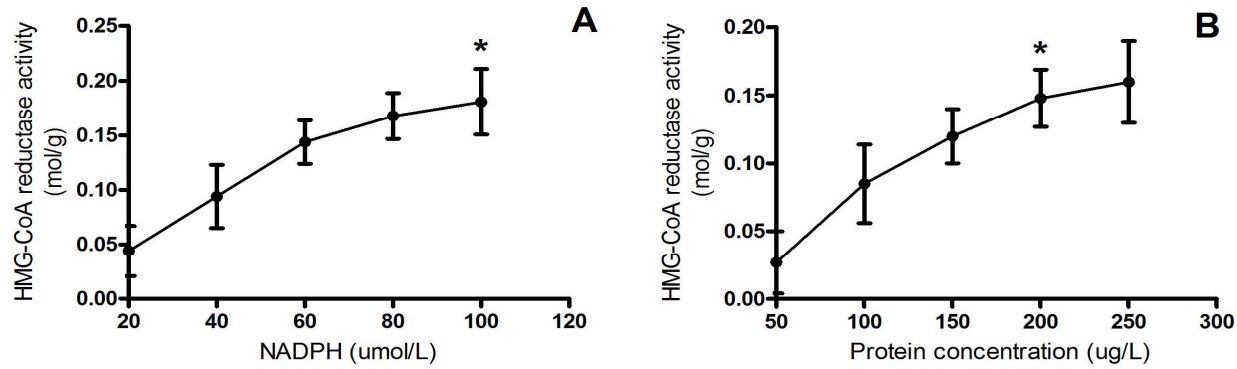

Figure 2: Effects of NADPH concentration (A) and microsomal protein concentration (B) on HMG-CoA reductase activity. Data points are mean ± SD (n = 5); $^{*}p < 0.05$ *vs* control group

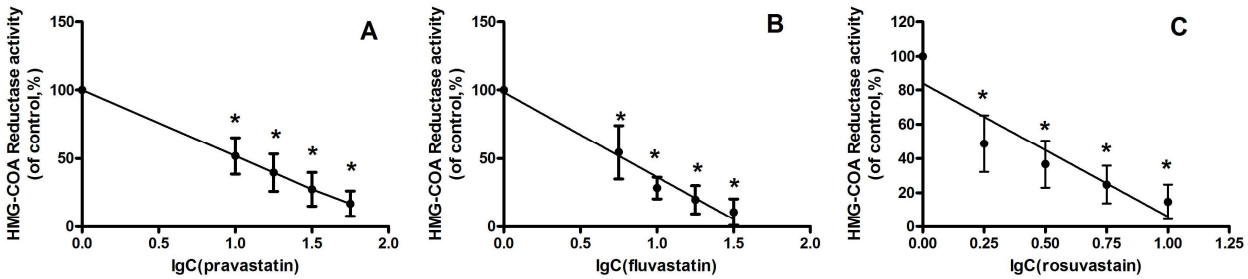

Figure 3: Pravastatin (A), fluvastatin (B), and rosuvastatin (C) inhibition of HMG-CoA reductase activity detected using the optimized spectrophotometric assay. Data points are mean ± SD (n = 5); $^{*}p < 0.05$ vs. control group

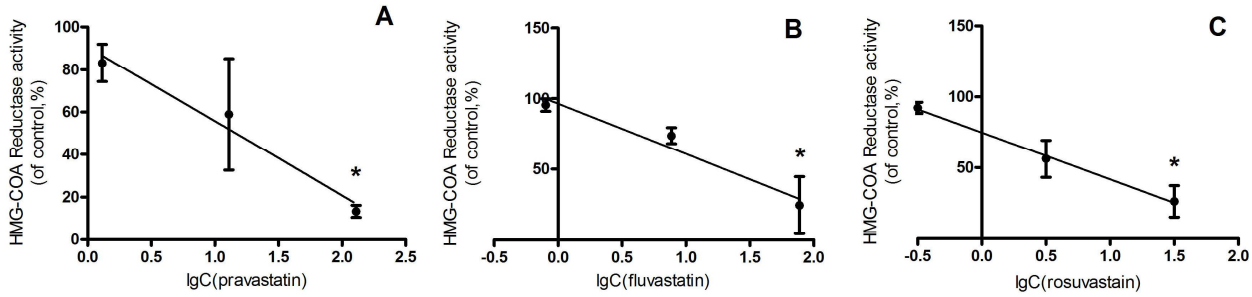

Figure 4: Inhibitory effect of pravastatin (A), fluvastatin (B), and rosuvastatin (C) inhibition of HMG-CoA reductase activity detected using a radioisotope assay. Data points are expressed as mean ± SD (n = 5); $^{*}p < 0.05$ vs. control group (pravastatin 1.3 μg/L, fluvastatin 0.7 μg/L, and rosuvastatin 0.3 μg/L)

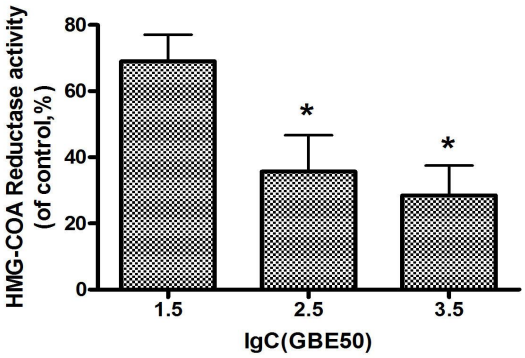

Figure 5: Effect of *Ginkgo biloba* extract (GBE50) on HMG-CoA reductase activity. Data points are expressed as mean ± SD (n = 5); $^{*}p < 0.05$ vs control group

We also evaluated the three inhibitors using a radioisotope assay. There was a linear relationship between HMG-CoA reductase inhibition and drug concentration for all three known inhibitors, and the r values were 0.985, 0.976, and 0.999, respectively (Fig 4). The IC_{50}s of pravastatin, fluvastatin, and rosuvastatin from the radioisotope assay were as follows: 0.0343, 0.0485, and 0.0119 μM, respectively.

Application of the optimal spectrophotometric assay

GBE50 inhibited HMG-CoA reductase activity in a concentration-dependent manner (Fig 5, $p < 0.05$).

DISCUSSION

Over the years, the major shortcomings of the spectrophotometric HMG-CoA reductase activity assay have been identified. Direct spectrophotometric NADPH oxidation determination is readily subject to interference by other NADPH-expending reactions, and these non-specific reactions produce excess background making the measurements inaccurate [15]. Rat liver microsomes, the enzyme source in this study, contain complex NADPH-dependent enzymatic systems that consist of HMG-CoA and others (e.g. HMG-CoA lyase) [16]. Therefore, it is important to improve the spectrophotometric accuracy by eliminating non-specific NADPH consumption that not derived from HMG-CoA. Previous studies obtained high HMG-CoA reductase activity using a complicated and expensive purification technique [17]. There is still a need for a simple and rapid HMG-CoA reductase activity assay.

In this study, we optimized several reaction variables including the reaction buffer pH, the preincubation and reaction duration, and substrate and microsomal protein concentrations. We employed the basic principles of optimization.

First, we adjusted each variable independently while keeping all others fixed to achieve the highest HMG-CoA reductase activity. Once the optimized variable was determined, it would be applied to the subsequent reaction system optimization experiments.

Our results showed that the optimal spectrophotometric HMG-CoA reductase activity assay conditions are as follows: reaction buffer pH 7.0, containing 100 μM NADPH and 200 μg/mL microsomal protein, incubating the protein and NADPH for 20 min at 37 ºC prior to 50 μM HMG-CoA addition, and reacting for 60 min at 37 ºC. High HMG-CoA reductase activity was obtained in these conditions. The HMG-CoA reductase specific activity was approximately 160 μmoL NADPH oxidized/min•mg microsomal protein under the optimal condition.

To validate this spectrophotometric assay system, we compared the kinetic results from our

optimized spectrophotometric assay with a radioisotope assay using three known HMG-CoA reductase inhibitors (pravastatin, fluvastatin and rosuvastatin). Our results showed that the IC_{50}s of the known inhibitors calculated using the improved spectrophotometric assay are the same order of magnitude as those obtained using a radioisotope assay. The GBE50 inhibition of HMG-CoA reductase activity was evaluated using the optimal spectrophotometric assay. GBE50 demonstrated concentration-dependent inhibition of HMG-CoA reductase activity. This result is consistent with our previously study using a radioisotope assay [18].

Recently, a HMG-CoA reductase assay kit became commercially available (Sigma-Aldrich, St. Louis, MO, USA). The kit provides the human enzyme catalytic domain (HMGR; recombinant GST fusion protein expressed in *E. coli*), and it has been used in some studies [19]. The assay also spectrophotometrically measures the absorbance decrease at 340 nm, which represents NADPH oxidation by HMGR in the presence of the HMG-CoA substrate.

However, according to the manufacturer's documentation, the kit is only designed to screen for different inhibitors/activators of the purified catalytic subunit *in vitro* (http://www.sigmaaldrich.com/china-mainland/zh/technical-documents/articles/biofiles/hmg-coa-reductase.html#Materials). *In vivo*, HMG-CoA reductase activity is controlled through synthesis, degradation, and phosphorylation in order to maintain the appropriate mevalonate-derived product concentrations. This regulation plays a vital role in hepatic lipid metabolism, especially for cholesterol metabolic pathway. Therefore, an accurate HMG-CoA reductase activity assay using whole protein from animals or tissues is imperative for investigating cholesterol homeostasis disturbance mechanisms and associated diseases, such as hypercholesterolemia.

Unfortunately, a well-developed, simple, rapid, and accurate method for assaying HMG-CoA reductase activity in these types of samples has not yet been achieved. For example, Rao *et al* described an indirect HMG-CoA reductase activity assessment in liver tissue [20], which is still being used by many investigators. Therefore, because the optimized spectrophotometric HMG-CoA reductase activity assay presented here is based on rat liver microsomes, it offers a new alternative for assessing tissue-derived whole protein, which would be valuable for investigating cholesterol metabolism in liver tissue. In addition, by using different enzyme sources (i.e. commercial human or animal microsomes), this improved method could also be used to screen candidate HMG-CoA reductase inhibitors in a simple, rapid, and low cost manner.

CONCLUSION

An improved spectrophotometric HMG-CoA reductase activity assay has been successfully developed by optimizing different variables relating to the reaction conditions, including preincubation of the enzyme and cofactor prior to HMG-CoA addition. This optimal method is rapid, simple and relatively low cost. It overcomes the low specificity and sensitivity associated with existing spectrophotometric methods, while maintaining a relatively high HMG-CoA reductase activity and greatly increasing hypolipidemic agent screening efficiency. Therefore, this improved spectrophotometric assay provides an alternative technique for studying cholesterol metabolism and favorable HMG-CoA reductase inhibitor screening system *in vitro*.

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