TITLE
Rapid RP-HPLC assay of HMG-CoA reductase activity.

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ABSTRACT
Radioisotope-based and mass spectrometry coupled to chromatographic techniques are the conventional methods for monitoring HMG-CoA reductase (HMGR) activity. Irrespective of offering adequate sensitivity, these methods are often cumbersome and time-consuming, requiring the handling of radiolabeled chemicals or elaborate ad-hoc derivatizing procedures. We propose a rapid and versatile RP-HPLC method for assaying HMGR activity capable of monitoring the levels of both substrates (HMG-CoA and NADPH) and products (CoA, mevalonate and NADP⁺) in a single 20min run, with no pretreatment required. The linear dynamic range was 10-26 pmoles for HMG-CoA, 7-27 nmoles for NADPH, 0.5-40 pmoles for CoA and mevalonate, and 2-27 nmoles for NADP⁺, and LOD values were 2.67 pmoles, 2.77 nmoles, 0.27 pmoles and 1.3 nmols, respectively.
INTRODUCTION

Hydroxymethylglutaryl-coenzyme A reductase is the enzyme that catalyzes the four-electron reductive deacylation of HMG-CoA to CoA and mevalonate (Fig.1) (1). This reaction is the controlling step in the biosynthesis of sterols and isoprenoids (2-3), hence a large number of studies on the modulation of HMGR activity is continuously performed in the effort of developing new drugs in the treatment of hypercholesterolemic disorders (1).

HMG-CoA reductase activity is conventionally assayed using elaborate radiochemical assay (4-9), chromatographic techniques coupled with mass spectrometry (10-15), or spectrophotometrically by monitoring of the decrease in the absorbance of co-factor NADPH at 340nm (16).

Herein, as an alternative for laboratories with no access to the expensive LC/MS equipment, we propose a rapid and adequately sensitive HPLC-based method capable of monitoring both the levels of all the species involved in the equilibrium in a single analysis, and the kinetics of HMG-CoA reductase-catalyzed reactions.

MATERIALS AND METHODS

Reagents – HMG-CoA reductase, HMG-CoA, NADPH, NADP⁺, coenzyme A, potassium phosphate, sodium phosphate, magnesium sulphate, phenyl-methane-sulfonyl-fluoride (PMSF), tosyl-phenyl-alanyl-chloromethyl-ketone (TPCK), EDTA, DTT and DMSO were all purchased from Sigma-Aldrich. HPLC grade methanol was obtained from JT Baker. All solvents and reagents were of the highest purity available.

HPLC analysis – A HPLC system Gold (Beckman Coulter Inc.) equipped with a UV/VIS detector and HPLC column heater (Alltech), was used for the analysis. Reaction mixture consisting of HMGR (0.4 µM), NADPH (2.68 mM) and HMG-CoA (1.55 µM) diluted in the activity buffer was incubated at 37°C, and aliquots were withdrawn at indicated times, and separated by HPLC. Each species (both isolated analytes and incubation mixtures) were injected and separated on a reverse phase Phenomenex Luna C18 column (5 µm particle size, 250 × 4.6 mm equipped with a 5 mm guard column of the same phase) thermostated at 26°C, with the following linear gradient of potassium phosphate 100mM (solvent A) and methanol (solvent B): 10%-30% B up to 15 min and 30%-10% B in 5 min, at flow rate of 0.8 ml/min, UV/VIS detector set at 260nm. An injection volume of 10 µl was used throughout. After each chromatographic elution, column was regenerated with two column volumes of 60% methanol. HMG-CoA, NADPH and NADP⁺ were directly monitored, whereas mevalonate was determined by monitoring CoA production (mevalonate/CoA 1:1
stoichiometric ratio, Fig.1). Enzyme catalytic activity was performed at 37°C in a 100mM sodium phosphate buffer containing 1 mM EDTA, 10 mM DTT, 2% DMSO, 1 mM magnesium sulphate, pH 6.8, following the reaction for different times of incubation. No interfering signal was detected upon injection of the activity buffer. Each analysis was repeated in quadruplicate. Detection of the HMGR levels and activity in cell homogenate was carried out on human colon cancer cell line HCT116, previously shown to express HMGR (17). Cells were suspended in the activity buffer added with PMSF and TPCK as proteases inhibitors and lysated using a insulin syringe. Total cellular enzyme levels were quantified according to the standard addition method (18): different amounts of the isolated enzyme (30-130 ng per mg of total proteins) were added to the reaction mixture (NADPH, HMG-CoA and cell lysate, dissolved in the activity buffer) and detecting the product of CoA after 60 min. This mixture was finally centrifuged at 10000×g for 10 min. These quantities were normalized to the total cell lysate proteins, detected using Bradford assay (19) with bovine serum albumin as standard.

RESULTS AND DISCUSSION

Calibration curves were established for each single analyte over adequate ranges of concentrations (Fig.2). Dynamic ranges of linearity for the individual samples (determined by simple linear regression analysis), along with LODs and LOQs (determined as ratio between 3 and 5 times the RMSE of the linear fit and the slope of the calibration curve, respectively) are reported in table 1. The linearity of the standard curves was evaluated with a chi-square $\chi^2$ goodness of fit test ($p > 0.05$).

The substrates and the products of the enzymatic reaction were clearly and completely separated from each other, as shown in figure 3A.

Time course of mevalonate/CoA production was analyzed according to a both mono-exponential model (Fig.4), and bi-exponential analysis did not significantly improve the quality of the fit, as assessed with F-Test (95% confidence).

The assay reproducibility was evaluated by comparison of intra- and interday variability over 5 days by performing 4 replicates each day, with maximum variation coefficient associated to peak area $CV_{\text{intra}} = 5.48\%$ and $CV_{\text{inter}} = 6.31\%$, and to retention time $CV_{\text{intra}} = 0.14\%$ and $CV_{\text{inter}} = 0.23\%$.

The same method was successfully applied to a cellular lysate, reporting comparable chromatographic profiles without any significant interfering compounds (figure 3B).
Multiple standard additions of the isolated enzyme to reaction mixture containing cell lysate, showed a linear correlation between CoA/mevalonate produced and total enzyme. The measured concentration of the cellular active enzyme was $52 \pm 2$ ng per mg of total proteins (Figure 5), corresponding to $15.58 \pm 0.48$ units/mg cell protein and consistently with previously studies on tumour cells (9).

Finally, the proposed method was validated by comparison with a commercially available spectrophotometric assay kit (Cod. CS1090, Sigma-Aldrich), which provided comparable levels of HMGR in HCT116 cell lysates ($14.8 \pm 0.4$ units/mg of total cell proteins).

In conclusion, this method could represent an useful tool for both rapid and low-cost routine assays HMG-CoA reductase activity, and quantitation of all the species involved in the equilibrium.
LEGENDS TO FIGURES

Fig. 1 – Schematic representation of HMG-CoA reductase enzymatic reaction.

Fig. 2 – Calibration curves of CoA, NADPH, HMG-CoA and NADP⁺. Linear fits (solid lines) and 95% confidence bound (dotted lines) are reported. Each experimental point was the average of three replicates.
Fig. 3 – Panel A: representative chromatographic profiles of reaction mixture in the absence (dashed line) and in the presence (after 60 min of incubation) of HMG-CoA reductase (solid line). Panel B: chromatographic profiles of cell lysate in the absence (grey line) and in the presence of reaction mixture after 0 and 60 minutes of incubation (dashed and solid line, respectively). Methanol gradient is reported (dotted line).

Fig. 4 – Time course of CoA/mevalonate production fitted to mono-exponential model (solid line): 

\[ \text{CoA} = R_{\text{max}} \times (1 - e^{k \times \text{time}}) \]

with \( R_{\text{max}} = 4.5 \text{ pmol} \) (maximum equilibrium response at \([\text{HMG-CoA}] = 1.55 \text{ µM}\)) and \( k = 0.026 \text{min}^{-1} \) (pseudo-first order rate constant). Standard deviation to fit are reported.
**Fig. 5** – Linear dependence between isolated enzyme added and CoA/mevalonate produced in a cell lysate reaction mixture. Quantity of the enzyme present in the cell lysate (normalized by the total quantity of proteins) is derived from the intercept on the X-axis.

**TABLES**

<table>
<thead>
<tr>
<th>Analyte tested</th>
<th>Limit of detection (LOD)</th>
<th>Limit of quantification (LOQ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADPH</td>
<td>2.77 nmol</td>
<td>4.62 nmol</td>
</tr>
<tr>
<td>NADP⁺</td>
<td>1.33 nmol</td>
<td>2.22 nmol</td>
</tr>
<tr>
<td>HMG-Coenzyme A</td>
<td>2.67 pmol</td>
<td>4.45 pmol</td>
</tr>
<tr>
<td>Coenzyme A</td>
<td>0.27 pmol</td>
<td>0.45 pmol</td>
</tr>
</tbody>
</table>

**Table 1** – Limit of detection (LOD) and limit of quantification (LOQ) of the analytes tested with this RP-HPLC method.