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Analysis of 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors using liquid chromatography–electrospray mass spectrometry

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Abstract

Employing high-performance liquid chromatography–electrospray mass spectrometry, we describe a new assay for monitoring 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase activity. Incubations were carried out with HMG-CoA reductase (rat liver), HMG-CoA and NADPH, and terminated by the addition of HCl. The reaction product, mevalonolactone, and internal standard, were extracted with ethyl acetate, dissolved in methanol, and analyzed by LC–MS. Using an isocratic mobile phase of 10% acetonitrile and 0.1% formic acid (flow-rate, 0.2 ml/min), the protonated molecules of mevalonolactone at m/z 131 and internal standard, β,β-dimethyl-γ-(hydroxymethyl)-γ-butyrolactone, at m/z 145, were detected using selected ion monitoring. The limit of detection was approximately 6.5 pg, and the limit of quantitation was approximately 16.3 pg. Extraction recovery was >90%. The relative standard deviations for intra- and inter-day assays were approximately 4.1±2.7 and 9.4±3.4%, respectively. Mevalonolactone was examined over a period of 3 days and found to be stable. Using this assay, lovastatin and mevastatin inhibited HMG-CoA reductase activity with IC_{50} values 0.24±0.02 and 2.16±0.31 μ*M*, respectively. These methods offer some advantages over those reported previously which employ radiolabeled substrate and products, and should be useful in searching for compounds that could lower serum cholesterol or alter cell growth and differentiation.

Keywords: 3-Hydroxy-3-methylglutaryl-coenzyme A reductase

1. Introduction

3-Hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (EC 1.1.1.34) is the key enzyme in the biosynthesis of isoprenoids, producing intermediates such as farnesyl and geranylgeranyl pyrophosphates, which lead to products such as cholesterol and dolichol (Fig. 1) [1]. HMG-CoA reductase catalyzes the reaction HMG-CoA+2NADPH+$2H^+$→mevalonic acid+2$NADP^+$+CoASH. Inhibition of this reaction is especially noteworthy since reductions in serum cholesterol correlate with reductions in coronary heart disease and atherosclerosis [2,3], but inhibition may also be associated with chemotherapeutic or chemopreventive activities [4–8]. Cholesterol is an essential cell membrane com-

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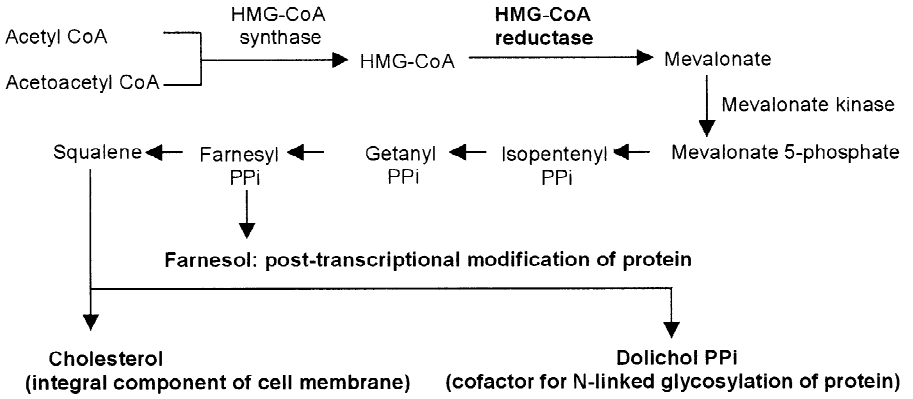

Fig. 1. Mevalonate pathway and main components that may effect cell growth and division. PPi, Pyrophosphate.

ponent required by mammalian cells for growth. Therefore, rapidly proliferating cancer cells may have a higher cholesterol requirement and some human hepatoma and colorectal cancer cells demonstrate elevated levels of HMG-CoA reductase activity [4]. In addition, isoprenoids have been suggested to serve as growth regulators, since they are substrates for farnesylation of the *ras* oncogene product [9]. Ultimately, increased HMG-CoA reductase activity may play a role in malignancy, and inhibition may have therapeutic value beyond treatment of hypercholesterolemia. For example, lovastatin [10–13], a representative inhibitor of HMG-CoA reductase, blocks farnesylation of Ras, and simvastatin [14,15], a hypocholesterolemic drug that competitively inhibits intracellular synthesis of mevalonate, displays a synergistic effect with carmustine (BCNU) with human glioma cells. Therefore, HMG-CoA reductase inhibitors are pharmacological tools of potential value for the control of abnormal cell growth.

A conventional assay method for assessing HMG-CoA reductase activity is based on preparative thin-layer chromatography, and [^{3}H]mevalonolactone and [^{14}C]HMG-CoA are employed for monitoring the reaction [16,17]. In addition, methods such as gas chromatography, paper chromatography, colorimetric methods, ion-exchange column chromatography, and gas chromatography–mass spectrometry have been devised [18–22]. The purpose of the present study was to develop a simple and specific method for assessing HMG-CoA reductase inhibitors. The procedure involved high-performance liquid chromatography–electrospray mass spectrometry (HPLC–ESI-MS), which proved to be a sensitive method for the detection and quantification of mevalonolactone.

2. Experimental

2.1. Reagents

HMG-CoA, NADPH, mevalonolactone, lovastatin, mevastatin and other reagents for the enzyme assay were purchased from Sigma (St. Louis, MO, USA). Solvents for HPLC were purchased from Fisher Scientific (Pittsburgh, PA, USA), and β,β-dimethyl-γ-(hydroxymethyl)-γ-butyrolactone was purchased from Aldrich (Milwaukee, WI, USA).

Stock solutions of mevalonolactone (10 m*M*) and β,β-dimethyl-γ-(hydroxymethyl)-γ-butyrolactone (10 m*M*) were prepared in distilled water and appropriate dilutions were made with water. Standard solutions of mevalonolactone were prepared for a calibration curve by adding various quantities of the stock solution to an inactivated reaction mixture (enzyme, substrate, buffer, and internal standard without cofactor) (see below).

2.2. HMG-CoA reductase assays

For the preparation of HMG-CoA reductase, female Wistar rats were purchased at 10 days of age (Harlan Sprague–Dawley, Indianapolis, IN, USA),

and maintained for 3 weeks on a controlled lighting schedule in which the room was illuminated from 18:00 to 06:00 daily. Rats were fed powdered diet (4% rat/mouse chow; Teklad, Madison, WI, USA) containing 2% cholestyamine resin for a minimum of 4 days prior to sacrifice during a mid-dark period [23].

Microsomal HMG-CoA reductase was prepared at 0–4°C using a modification of the method described by Parker et al. [24] as follows. Rat livers were immediately excised, washed twice in buffer A [50 m*M* imidazole–HCl, pH 7.2, 50 m*M* NaCl, 250 m*M* sucrose, 10 m*M* EDTA, 10 m*M* ethylene glycol-bis(β-amino ethyl ether)*N,N,N′,N′*-tetraacetic acid (EGTA), 5 m*M* dithiothreitol (DTT), and 50 μ*M* leupeptin], homogenized (2 ml buffer A per gram of liver) and centrifuged (16 000 *g*, 10 min). The centrifugation step was repeated with the supernatant, and the postmitochondrial supernatant solution was centrifuged at 140 000 *g* for 75 min. The resulting supernatant solution was decanted, and the material remaining on the wall of the ultracentrifugation tube was suspended in buffer B (50 m*M* imidazole–HCl, pH 7.2, 250 m*M* NaCl, 1 m*M* EDTA, 1 m*M* EGTA, 5 m*M* DTT and 20 μ*M* leupeptin). Protein content was determined by the method of Lowry et al. [25], and samples were stored at −80°C.

For HMG-CoA reductase assays, microsomes (200 μg protein) were preincubated with buffer B for 5 min at 37°C, and the reaction was initiated by adding 50 μl of cofactor–substrate solution (240 nmol NADPH and 40 nmol HMG-CoA in buffer B). Following incubation at 37°C for the specified time, the reaction was terminated by the addition of 10 μl of 6 *M* HCl and incubation for an additional 40 min at 37°C to promote the generation of mevalonolactone. After centrifugation to remove precipitated protein (13 000 *g*, 2 min), the supernatant was neutralized with sodium carbonate buffer (pH 10.5). Internal standard was added, and the supernatant was extracted four times with five volumes of ethyl acetate. Vials were centrifuged for phase separation (13 000 *g*, 2 min), the pooled EtOAc fractions were evaporated in a vacuum centrifuge at room temperature, and the residue was re-dissolved in 200 μl of methanol for LC–MS analysis. All samples were tested in duplicate. Lovastatin was used as a positive control, and negative controls were prepared by replacing NADPH with reaction buffer.

2.3. LC–MS

The LC–MS system consisted of a Hewlett-Packard (Palo Alto, CA, USA) 5989B mass spectrometer with an Analytica (Bradford, CT, USA) electrospray interface, and a Hitachi HPLC system (Tokyo, Japan) with L-7100 pumps, an L-7400 UV detector, and an L-7250 programmable autosampler.

The HPLC separations were carried out using a Hewlett-Packard (Wilmington, DE, USA) ODS Hypersil (100×2.1 mm I.D.) column with 5 μm packing material. Aliquots (5 μl) of each sample were directly injected onto the column and eluted with 10% acetonitrile containing 0.1% formic acid at a flow-rate of 0.2 ml/min. The eluent was introduced to the electrospray interface without splitting. The nitrogen nebulizing gas was 80 p.s.i. (1 p.s.i.= 6894.76 Pa), and the flow-rate of the nitrogen drying gas was 40 ml/min at 300°C. The entrance lens and capillary exit voltages were set at 23 and 86 V, respectively. Source temperature was maintained at 120°C. Total ion scan of mevalonolactone was performed in the range of m/z 70–200 with a scan rate of 0.5 scans/s. Capillary and multiplier voltages were set at 3000 and 2100 V, respectively. The protonated molecules of mevalonolactone and internal standard were observed by selected ion monitoring (SIM) with a dwell time of 500 ms.

Sample recovery was determined by calculating the concentrations of mevalonolactone in spiked samples against calibration curves. Calibration curves were generated by using mevalonolactone standard solutions (0.04, 0.1, 0.2, 0.4, 1 and 2 μmol) prepared in 100 μl of inactivated reaction mixtures containing internal standard (0.1 μmol). These solutions were extracted with ethyl acetate and then evaporated to dryness and re-dissolved in 200 μl of methanol. Experiments were repeated five times. Solvent extraction recovery of mevalonolactone was compared with that from non-extracted sample. Intra- and inter-day precisions were studied over 3 different days with three concentrations of mevalonolactone (0.1, 0.2 and 1 μmol). Analyses were performed as described above.

3. Results and discussion

The positive ion electrospray mass spectrum showed protonated molecules of mevalonolactone at m/z 131 and a base peak at m/z 113 which corresponded to loss of water from the protonated molecule (Fig. 2). No abundant sample ion was detected using negative ion electrospray (data not shown). The internal standard, β,β-dimethyl-γ-(hydroxymethyl)-γ-butyrolactone, produced a base peak at m/z 145 corresponding to the protonated molecule in positive mode. Therefore, HMG-CoA reductase activity was measured using positive ion electrospray mass spectrometry with SIM of the ions m/z 131 and 113 for mevalonolactone and m/z 145 for the internal standard. Mevalonolactone and β,β-dimethyl-γ-(hydroxymethyl)-γ-butyrolactone produced symmetrical peaks during reversed-phase HPLC, with elution times of 2.8 and 4.3 min, respectively (Fig. 3).

For the improvement of mevalonolactone detection limits and to eliminate salts and interfering signals, mevalonolactone was extracted from aqueous buffer using ethyl acetate. The extraction efficiency was 92.1±2.1%. With negative controls in which NADPH was replaced with reaction mixture, mevalonolactone was not observed in the mass chromatogram. The specificity of the assay was further confirmed since mevalonolactone formation decreased in the presence of a positive control, lovastatin (Fig. 3). As shown in Fig. 4, the concentration of mevalonolactone increased linearly for approximately 10 min and reached a plateau between 10 and 30 min. The effect of dimethylsulfoxide

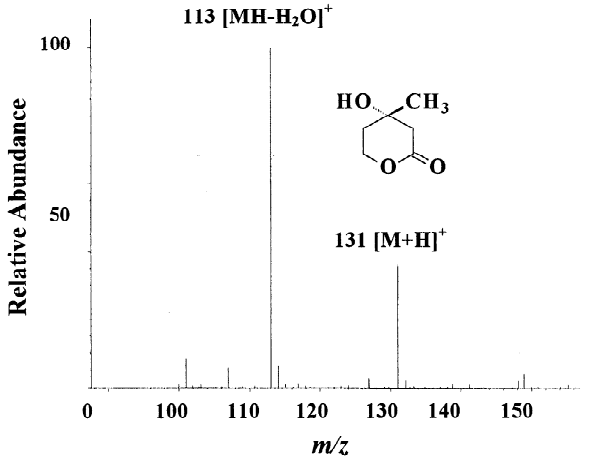

Fig. 2. Positive ion electrospray mass spectrum of mevalonolactone.

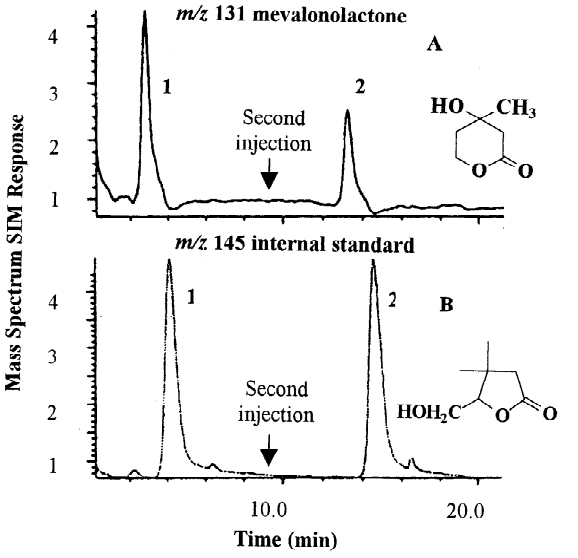

Fig. 3. Positive ion electrospray LC–MS with SIM analysis of (A) mevalonolactone and (B) internal standard. Extracts were obtained from a control incubation (peak 1 in A and B) and an incubation containing lovastatin (0.4 μg/ml) (peak 2 in A and B). Serial injections were used for these two analyses.

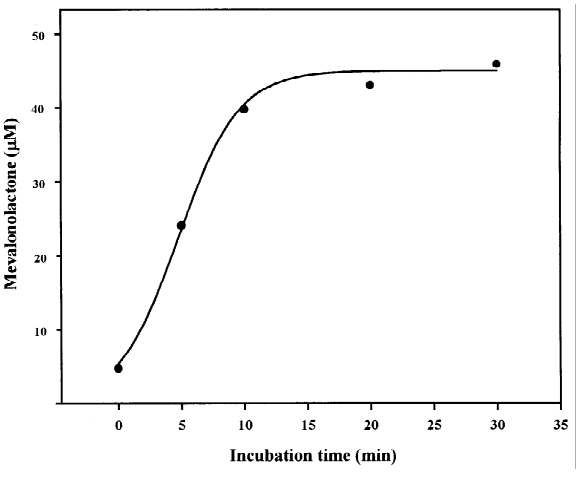

Fig. 4. Effect of incubation time on HMG-CoA reductase activity. HMG-CoA reductase assays were carried for 0, 5, 10, 20 and 30 min; for the zero-time point, cofactor–substrate and HCl were added together.

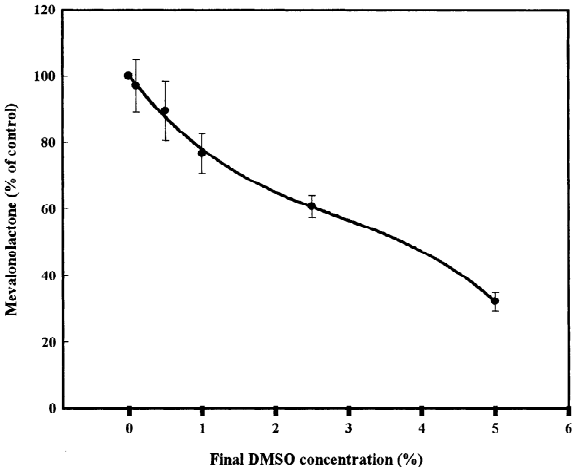

Fig. 5. Effect of DMSO on HMG-CoA reductase activity. Assays were performed as described in Experimental in the presence of 0.1, 0.5, 1, 2.5 or 5% DMSO. The resulting quantities of mevalonolactone were compared with a control sample which did not contain any DMSO (100% value).

(DMSO), a solvent of general utility for the dissolution of test compounds, was also studied, and concentrations below 0.5% were found to permit enzyme activity above 90% (Fig. 5).

The limits of detection (LOD) and quantitation (LOQ) for mevalonolactone were determined at signal-to-noise ratios (S/N) of ≥ 3 and ≥ 10, respectively, and were 6.5 and 16.3 pg, respectively (Fig. 6). The inter- and intra-day relative standard deviations (RSDs) of mevalonolactone were 9.4% (range 5.5–11.3%) and 4.1% (range 1.0–6.0%), respectively, as determined with five replicate samples. A calibration curve consisting of six calibration points produced a slope of 0.432, an intercept of 0.8007, and the correlation coefficient was >0.995. Recovery of mevalonolactone from inactivated reaction mixture compared with water solution was $94.8 \pm 4.3\%$. To determine the stability of mevalonolactone as a function of time, the same samples were tested over a period of 3 successive days. Mevalonolactone was found to be stable for this period (data not shown).

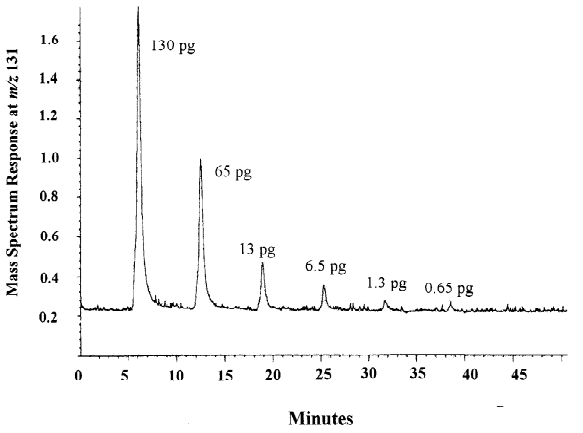

Fig. 6. Limit of detection of mevalonolactone using LC–MS with SIM. The indicated quantities of mevalonolactone were applied by serial injection and analyzed.

The inhibitory activity of test samples was determined by comparing the peak areas of mevalonolactone in selected ion chromatograms. Relative to controls, lovastatin and mevastatin inhibited the production of mevalonolactone in a concentration-dependent manner, with IC_{50} values of 0.24 ± 0.02 and 2.16 ± 0.31 μM, respectively (Fig. 7).

In conclusion, the method described herein appears to be adequate for monitoring the activity of

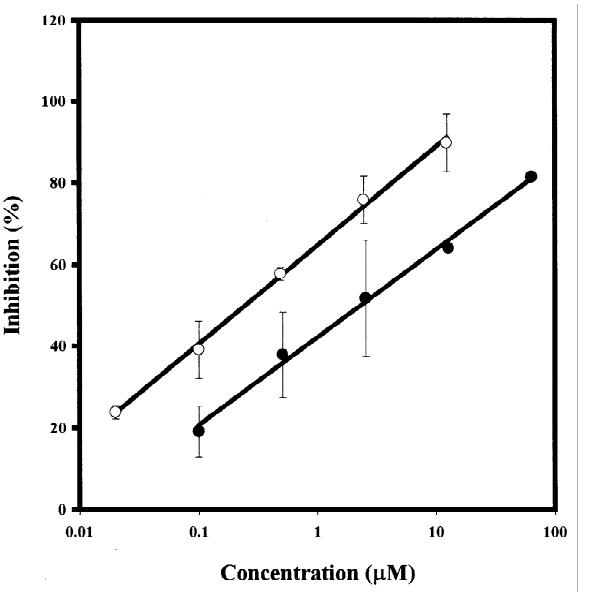

Fig. 7. Inhibition of HMG-CoA reductase by lovastatin and mevastatin. The indicated concentrations of lovastatin (○) or mevastatin (●) (dissolved in DMSO) were preincubated with enzyme (10 min), and then incubated for an additional 20 min following the addition of cofactor–substrate mixture (final DMSO concentration, 0.1%). Product was analyzed by LC–MS as described in the Experimental. Each concentration was analyzed in duplicate.

HMG-CoA reductase. One advantage of this LC–MS method compared to previous methods is that the use of radiolabeled materials is not required. In addition, the method is rapid, precise, and accurate, and detection and quantification may be less cumbersome than conventional assays. Since rapid isocratic HPLC separations are used, the assay can be performed in a manner that permits high throughput for the evaluation of potential inhibitors.

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