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Measurement of HMG CoA reductase activity in different human cell lines by ultra-performance liquid chromatography tandem mass spectrometry

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ABSTRACT

Hydroxymethylglutaryl coenzyme A reductase (HMGCR) catalyzes the rate limiting step in cholesterol biosynthesis converting HMG-CoA into mevalonic acid (MVA), which equilibrates with mevalonic acid lactone (MVL) under neutral pH conditions.

We developed a fast, sensitive, and efficient method to determine HMGCR activity in human cell lines measuring MVL levels by ultra-performance liquid chromatography tandem mass spectrometry (UPLC–MS/MS). Convenient prepared samples containing MVL-D_7 as an internal standard were injected, separated, and eluted from an ACQUITY HSS PFP column. Measurement of MVL was performed by electrospray ionization mass spectrometry with multiple reaction monitoring. Calibration curves were linear and reproducible in the range of 0.15–165 μg/l ($r > 0.99$). Lower limit of quantification was 0.12 μg/l. Intra- and interassay imprecision were <1.3% and <2.9%, respectively.

HMGCR enzymatic activity measurements of cells cultivated under different cell culture conditions (with 10% FCS, with 10% lipoprotein-deficient serum and under serum starvation) revealed the applicability of this test system for various experimental settings.

This efficient UPLC–MS/MS assay permits rapid and high sensitive determination of HMGCR enzyme activity, tracing potential alterations in cholesterol biosynthesis.

1. Introduction

Principles of cholesterol biosynthesis were discovered by Bloch and Lynen in the 1940s, who received the Nobel Prize for Physiology or Medicine in 1964 [1]. Multiple human disorders are linked to enzyme defects based on genetic mutations, leading to abnormal regulation and intermediate accumulation in cholesterol biosynthesis [2,3]. Cholesterol serves as an essential compound giving fluidity and permeability to membranes. Furthermore, cholesterol biosynthesis provides sufficient amounts of isoprenoids, bile acids and several steroids to the human body [4]. However, high cholesterol levels in blood are associated with an increased risk of cardiovascular disease, including myocardial infarction and stroke. Cholesterol biosynthesis, localized in the endoplasmic reticulum (ER), starts with the rate limiting enzyme hydroxymethylglutaryl coenzyme A reductase (HMGCR), whose activity is strongly controlled by several feed-back mechanisms involving endogenous pathways and exogenous cholesterol intake by nutrition [5–7]. The reduction of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) by HMGCR forms mevalonic acid (MVA), which equilibrates with its lactone form (MVL) under neutral pH conditions (Fig. 1). Beg et al. [8] showed that the short time regulation of HMGCR by phosphorylation/dephosphorylation status permits a rapid response in cholesterol biosynthesis to altered metabolic conditions. Withdrawal of fetal calf serum (FCS) or culture conditions in lipoprotein-deficient serum confirmed that HMGCR activity could be quickly induced in human dermal fibroblasts [5,9,10]. A couple of methods have been established measuring HMG CoA reductase activities in liver slices and cell cultures, as well as serum, plasma and urine MVA/MVL levels, using radioisotope (RI) techniques [11,12], enzyme immunoassays [13] and chromatography coupled mass spectrometry [14]. Radioenzymatic assays can be classified into HMGCR enzyme activity measurements using ^{14}C-HMG-CoA to form ^{14}C-mevalonic acid [15] and direct measurements of 5-[^{32}P] phospho-MVA in plasma [11]. However, in terms of irradiation risks and raising availability of stable isotope labeled standards, RI techniques might not compete against improved measurement accuracies using mass spectrome-

Abbreviations: UPLC–MS/MS, ultra-performance liquid chromatography tandem mass spectrometry; LLOQ, lower limit of quantification; MVL, mevalonic acid lactone; MVA, mevalonic acid; HMGCR, hydroxymethylglutaryl coenzyme A reductase; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA.

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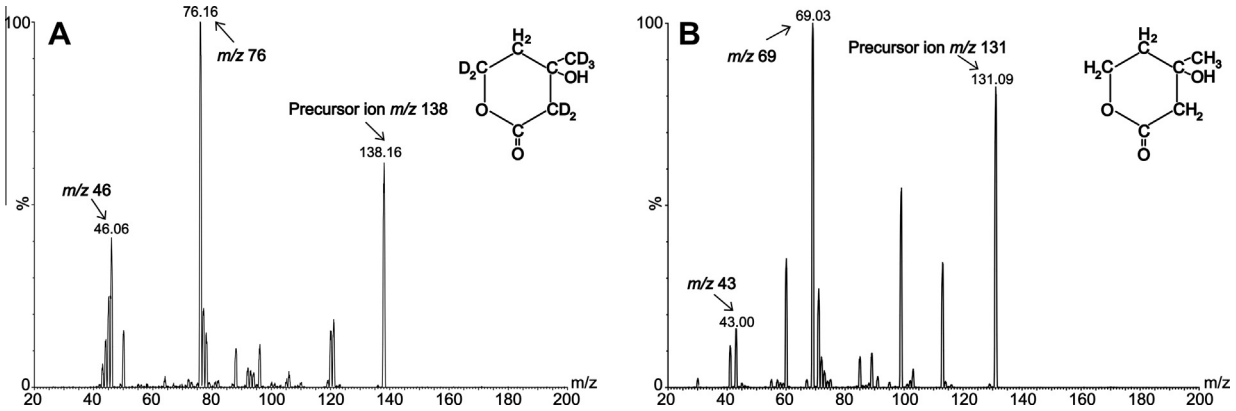

Fig. 1. Multiple reaction monitoring (MRM) mode of mevalonolactone (A) *m/z* 131 → 69 and *m/z* 131 → 43 for MVL. (B) *m/z* 138 → 76 and *m/z* 138 → 46 for the internal standard (MVL-D_7). Second mass transitions were used for analysis control.

try applications in the future. They provide accurate detection of analytes, even for small molecular weight compounds like MVA or MVL, scanning in multiple reaction monitoring (MRM) for specific mass transitions [16]. Anyhow, sample preparation and clean-up procedures remained difficult and time-consuming requiring derivatization steps [17], liquid–liquid [18], or solid phase extractions (SPE) [16].

Here we describe for the first time a convenient, sensitive and rapid method for the detection of mevalonic acid lactone by UPLC–MS/MS estimating HMGCR enzyme activity in different experimental cell culture settings.

2. Materials and methods

2.1. Reagents, internal standards, and calibrators

LC/MS-grade water, acetonitrile and methanol were purchased from Fisher Scientific GmbH (Schwerte, Germany). MVL, hydrochloric and formic acid were obtained from Sigma–Aldrich (Deisenhofen, Germany). MVL-D_7 (purity > 98%) was purchased from CDN Isotopes Inc. (Quebec, Canada).

A standard stock solution of MVL and MVL-D_7, each at concentrations of 1 mg/ml, were prepared separately in methanol/water and stored at −80 °C. Several calibrators (0.3125, 0.625, 1.25, 2.5, 5.0, 10.0, 20.0, 40.0, 80.0 μg/l) of MVL were prepared by dilution of the stock solution in enzyme reaction buffer (see Section 2.3.) containing 500 μg/ml recombinant human albumin from rice (Sigma–Aldrich, Deisenhofen, Germany) as equivalent sample matrix to cell lysates.

2.2. Cell culture

HepG2 (HB-8065), HEK-293 (ACC305) cells were purchased from ATCC and DSMZ, NHDF (M45D) were obtained from Genlantis. Cells were cultivated in a CO_2 incubator at 37 °C using 100-mm cell culture dishes (Greiner bio-one, Frickenhausen, Germany) containing 10 ml Dulbecco's modified essential medium (DMEM; Gibco) with 10% fetal calf serum (PAN Biotech, Aidenbach, Germany), 1% L-glutamine (200 mM) and 1% antibiotic/antimycotic solution (PAA, Pasching, Austria). For experiments cells were grown for 24 h in 10% FCS (177 cells/mm^2, BD Falcon), washed twice with phosphate-buffered saline (PBS; Gibco), and replaced with either 10% of fetal calf serum (FCS), lipoprotein-deficient FCS (LPDS) [10], or without serum for additional 24 h. Biological samples were prepared in triplicates. After stated time of growth, cells were washed twice with ice cold PBS and harvested by scraping into a microcentrifuge tube. Cells were pelleted by centrifugation (5 min, 3000*g*), whereupon the cell pellet was resuspended in 120 μl lysis buffer, containing 137.5 mM NaCl, 50 mM Tris/HCl (pH 7.8), 8.7% Glycerin, 0.5 mM EDTA (pH 8.0), 1% Protease Inhibitor (P2714; Sigma–Aldrich, Deisenhofen, Germany), 1% NP-40 (Sigma–Aldrich, Deisenhofen, Germany). Cell lysates were frozen at −80 °C for 1–2 h, again clarified by centrifugation (10 min, 8000*g*), aliquoted and stored at −80 °C prior to enzyme analysis or assayed immediately. Total protein content of cell lysates was estimated using bicinchoninic acid assay (BCA Kit, Sigma–Aldrich, Deisenhofen, Germany).

2.2.1. Enzyme activity assay

Preparation of enzyme activity assay was conducted according to Honda et al. [17] with modifications using 50 μg total protein of cell lysates, diluted in enzyme reaction buffer up to 100 μl in a 1.5-ml polypropylene microcentrifuge tube. The initial substrate HMG-CoA (200 μM) was diluted in enzyme reaction buffer (100 mM potassium phosphate buffer (pH 7.4), 0.1 mM EDTA, 50 mM KCl, 10 mM DTT, containing a NADPH generating system: 2.5 mM NADPH, 15 mM glucose-6-phosphate, and 1 unit of glucose-6-phosphate dehydrogenase) achieving a final reaction volume of 150 μl. Samples, blank samples or calibrators were incubated for 30 min at 37 °C with substrate mix, whereupon reaction was stopped by the addition of 20 μl HCl (6 N) to ensure the lactonization of mevalonic acid into MVL [17].

2.3. Sample preparation for UPLC–MS/MS

Samples, blank samples or calibrators of 150 μl were treated with 300 μl acetonitrile for protein precipitation, containing internal standard (MVL-D_7, 10 μg/l). The mixture was vortex-mixed for 5 s and centrifuged at 13,000*g* for 10 min, whereupon supernatants were transferred into another microcentrifuge tube and evaporated in a vacuum concentrator at RT for approx. 1.5 h (S-Concentrator BA-VC-300H; H. Saur Laborbedarf, Reutlingen, Germany). Residues were dissolved by vortex-mixing in 50 μl HPLC-grade water/methanol/acetonitrile containing 0.5 N HCl (1:1:1, v/v) for 10 s and centrifuged at 13,000*g* for 10 min. Supernatants were transferred to the autosampler vessels, whereupon a volume of 1 μl was injected into the UPLC–MS/MS system.

2.4. UPLC–MS/MS analysis

Separation of mevalonic acid lactone by an ultra-performance liquid chromatography (UPLC) system (Waters Acquity UPLC) was achieved by using a 2.1 mm × 150 mm HSS PFP UPLC/MS cartridge (Waters, 1.8 μm ACQUITY UPLC HSS PFP Column) conducted at 45 °C, which was directly coupled to a Quattro LC tandem mass spectrometer equipped with Z Spray ion source (Waters Xevo™

TQ-S). Flow rate was set at 0.2 ml/min. The gradient program was adjusted to 93%/7% water/methanol containing 0.1% formic acid and 2 mmol/l ammonium acetate for 2.5 min, followed by a linear gradient of 98% methanol containing 0.1% formic acid and 2 mmol/l ammonium acetate. After 3.5 min, the mobile phase was readjusted to 93%/7% water/methanol, terminating the run at 7 min. The mass spectrometer, performed in electrospray positive ionization mode, was controlled by using MassLynx V4.1 software, with automated data processing by the TargetLynx program. Nitrogen and argon were used as nebulizing and collision gas, respectively.

Instrument settings were set as follows: capillary voltage, 1 kV; source temperature, 150 °C; desolvation temperature, 500 °C; sample cone energy for MVL and MVL-D_7 was adjusted at 18 eV; and collision energy for MVL and MVL-D_7 at 8 eV. Multiple reaction monitoring (MRM) mode was used for sample analysis, with mass transitions of m/z 131 → 69, m/z 138 → 76 for MVL and MVL-D_7, respectively. Second mass transitions of MVL and its internal standard, m/z 131 → 43 and m/z 138 → 46, were used for analysis control.

2.5. Validation

This UPLC–MS/MS assay was validated according to STARD (Standard for Reporting of Diagnostic Accuracy) [19,20], whereby method properties like linearity, lower limit of quantification (LLOQ) and intra- and interday imprecisions were determined.

2.5.1. Linearity studies

A calibration curve for mevalonic acid lactone was generated with a collection of 16 different calibrators, prepared by serial dilution in enzyme reaction buffer (see Section 2.3.) containing 500 μg/ml of recombinant human albumin (Sigma–Aldrich, Deisenhofen, Germany) as equivalent sample matrix to cell lysates, in a range between 0.005 and 160 μg/l. Calibrators were analyzed in 4 replicates using the arithmetic average for determination of the lower limit of quantification (see Section 2.5.2), as well as single measurement values for linearity definition.

2.5.2. Lower limit of quantification (LLOQ)

The lower limit of quantification (LLOQ) was defined as the lowest concentration of the analyte at which the coefficient of variation (CV) was <20% (see Section 2.5.1).

2.5.3. Intra- and interday imprecision

The intraassay imprecision was ascertained by analyzing 10 replicates of samples (HepG2) with different MVL concentrations [low (4.4 μg/l), medium (16.7 μg/l) and high (32.2 μg/l)] on the same day. Additionally, the same samples were analyzed for interassay inaccuracy on 5 different days.

2.6. Statistics

Experimental data are indicated as means ± SD. Graphic data processing and statistics were performed with GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, USA), using simple linear regression and Kruskal–Wallis test with Dunn's post-test for statistical data analysis.

3. Results and discussion

3.1. UPLC–MS/MS analysis

We developed a fast and sensitive method for determining levels of mevalonolactone (MVL) by UPLC–MS/MS, enabling the investigation of numerous questions regarding cholesterol biosynthesis in cell culture systems.

Measurements of MVL and its deutered internal standard were carried out in multiple reaction monitoring (MRM) mode, using the most sensitive mass transitions for instrument adjustments and sample analysis. Molecule fragments were analyzed applying varying amounts of collision energy. Precursor ions $[M]^+$ of MVL and MVL-D_7 at m/z 131 and 138 were fragmented with a collision energy of 8 eV, obtaining the most sensitive and specific product ions at m/z 69 and 76 respectively (Fig. 1).

Chromatographic separation of MVL and MVL-D_7 by UPLC was performed using a 2.1 mm × 150 mm HSS PFP UPLC/MS cartridge, maintained at 45 °C. A precise and clear separation was detected at retention times of 4.86 min for MVL and MVL-D7, using an injection volume of 1 μl. Whole running-time was adjusted to 7 min. A representative chromatogram generated from a sample containing 73 μg/l MVL, as well as the internal standard MVL-D_7 (at a concentration of 10 μg/l), is shown in (Fig. 2). Other chromatographic options were critically monitored during our method development, using different kinds of UPLC-columns (Waters, 1, 7 μm ACQUITY BEH Phenyl column; Waters, 1, 7 μm ACQUITY CSH Fluoro-Phenyl column), chromatographic gradients, injection volumes or column temperature settings.

This method permits convenient sample preparation, without any derivatization steps or solid phase extractions, compared to published protocols [21,16]. Derivatization procedures, as described previously [17] or with other reagents [e.g. (2-Aminoethyl) trimethylammonium chloride hydrochloride, p-Iodo-DL-phenylalanine, 3-(Aminomethyl)-2-bromothiophene], achieved no higher sensitivity in our experiments than measuring MVL itself (data not shown). In addition, omitting derivatization steps might accelerate chromatographic separation due to a smaller molecule mass, or enlarge sample reproducibility and comparability, considering reaction efficiencies between different sample preparations. Furthermore, solid phase extractions as described (IST-ENV+; 100 mg/3 ml, Agilent Technologies) [22] were tested to ensure the best detection of MVL in samples. Nevertheless, basic protein precipitation with acetonitrile was chosen for its highest quality in chromatographic separation, peak constitution and sensitivity, as well as in comparison to utilize methanol or ether extractions (data not shown). A threefold concentration of sample supernatants, using vacuum dehydration, was performed to improve detection rates, whereupon a high sample throughput can be prepared contemporaneously.

3.2. Validation

Calibration curves covering 12 calibrators measured in 4 replicates for the analysis of MVL were linear and reproducible in the range of 0.15–165 μg/l ($r > 0.99$). The lower limit of quantification determined by the measurement of 12 calibrators using four replicates was 0.12 μg/l. Intraassay imprecisions (measured for MVL concentrations between 4.4 μg/l and 32.2 μg/l) were <1.3%, whereupon between-run imprecisions (measured on five different days) were 2.9% for the lowest, 2.1% for the mean and 2.0% for the highest MVL concentration.

3.3. HMGCR activity in various cell culture conditions

Three different cell types were tested for their HMG CoA reductase activity under different cell culture conditions, altering FCS supplementation.

Cell lysis was performed quickly on ice to avoid protein degradation, considering a high quality and entity of transmembrane protein content. Enzyme buffer composition, adapted from Honda et al. [17], was modified in NADPH and glucose-6-phosphate

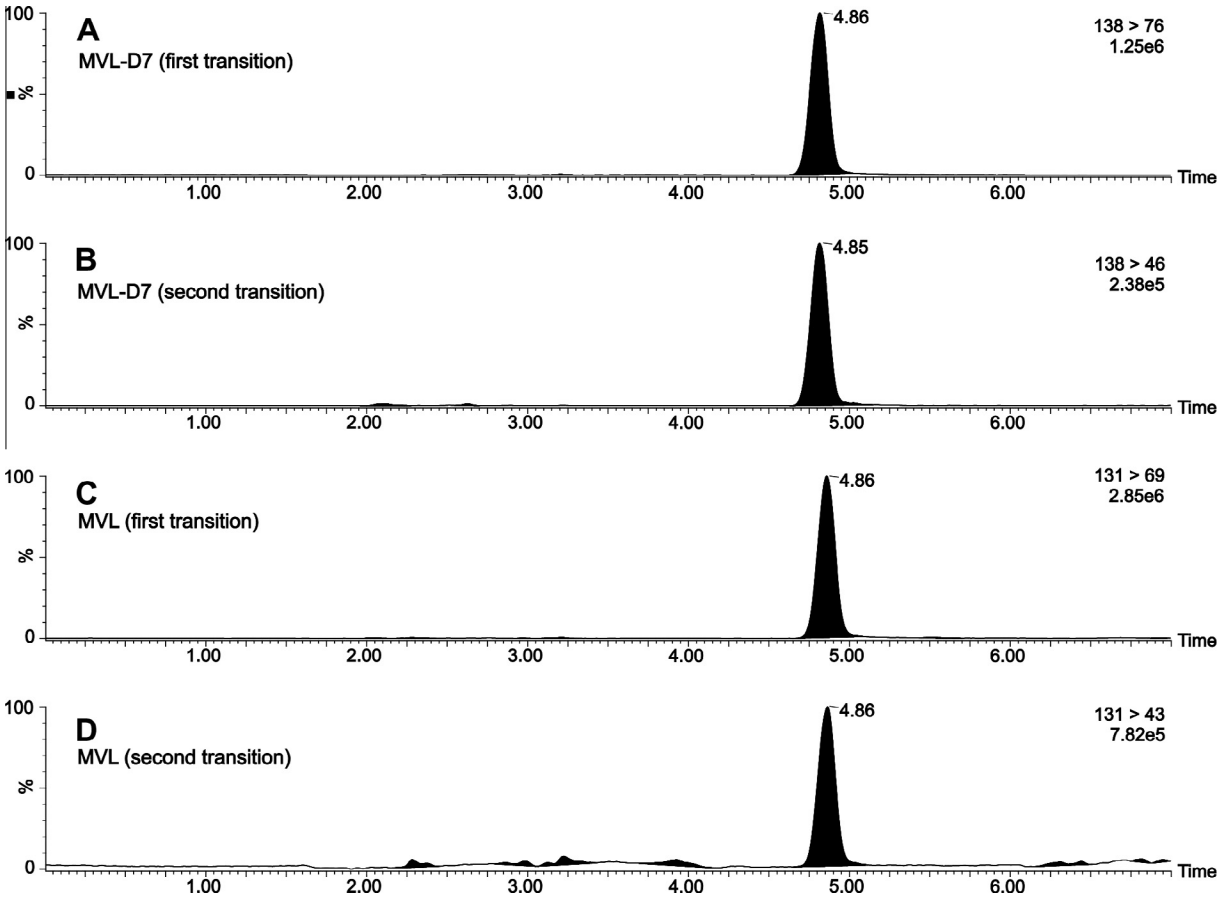

Fig. 2. Representative chromatograms of mevalonolactone (MVL) and its deuterated internal standard (MVL-D_7) in a sample of HepG2 cells converted HMG-CoA to 73 μg/l of MVL during enzymatic reaction (monitored in MRM).

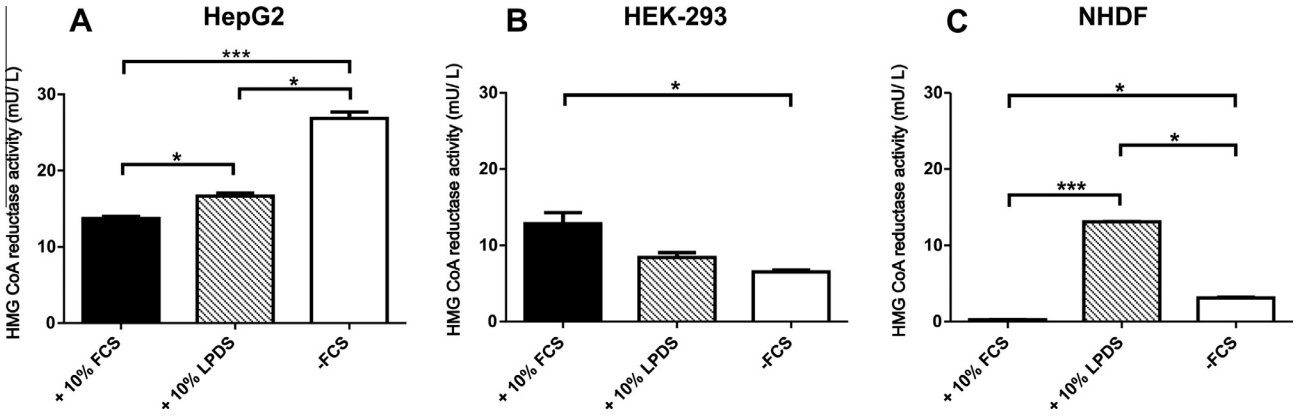

Fig. 3. Activity measurements of HMG-CoA reductase in (A) HepG2-, (B) Hek-293-cells and (C) NHDFs (M45D) grown for 24 h under different cell culture conditions (+10% FCS, +10% LPDS, -FCS). Data are presented in arbitrary units as means with corresponding standard error. Differences between cell culture settings were analyzed using Kruskal–Wallis test with Dunn's post-test (*$p < 0.05$; ***$p < 0.0001$).

concentrations. Especially high NAPDH concentrations decreased test sensitivity due to a high impact on ion suppression. Hence, we tested different concentrations of NADPH in the enzyme assay (34, 15, 10, 5, 2.5, 1.25 mM), to choose the lowest amount with no impact on enzyme activity measurements (data not shown). However, enzyme reaction systems described so far, varied remarkable in their compositions and concentrations [17,23,24]. Substrate reaction was carried out with 200 μM of HMG CoA, whereupon enzyme kinetic studies with increasing concentrations of HMG CoA revealed enzyme saturation under these conditions (data not shown).

Enzyme reactions were observed in all tested cell lines, varying in their magnitudes (Fig. 3). As shown in (Fig. 3), activity measurements for HepG2 cells revealed that serum starvation had the greatest impact on HMG CoA enzyme activity (Fig. 3a) increasing up to 26.6 mU/l. However, lipoprotein deprivation had little effect on cholesterol biosynthesis in HepG2 cells. HEK-293 cells exhibit the highest enzyme activity rates under cultivation with 10% FCS,

at which it decreased under delipidation and serum withdrawal (Fig. 3b). A contradictory activity pattern was observed in NHDF, which showed a 50-fold increase in HMG CoA reductase activity under cultivation with lipoprotein-deficient serum (Fig. 3c). In contrast, almost no enzyme reaction was detected under incubation with 10% of FCS.

Cell culture settings, regarding serum supplementation, or lipoprotein-deprivation had great impact on HMGCR activity in HepG2 and NHDF. As expected, liver cells (HepG2) exhibit the highest activity rates, at which HMGCR can be as well strongly induced by lipoprotein deprivation in human dermal fibroblasts, as described before [5][9]. These measurements demonstrate the applicability of this enzymatic test system for different experimental settings.

4. Conclusion

In summary, we developed an efficient, simple and sensitive method for measuring HMG-CoA reductase activities in different human cell lines, monitoring MVL levels by UPLC–MS/MS. This assay can be applied for various cell culture settings regarding cholesterol biosynthesis, e.g. investigating hypercholesterolemia, development of atherosclerosis, or statin efficiency control. It provides a convenient and practicable tool for laboratory use and research applications.

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References

[1] R. Zetterström, The 1964 Nobel Prize for the discovery of the biosynthesis of cholesterol, Acta Paediatr. 98 (2009) 1223–1227.
[2] F.D. Porter, G.E. Herman, Malformation syndromes caused by disorders of cholesterol synthesis, J. Lipid Res. 52 (2011) 6–34.
[3] H.R. Waterham, Defects of cholesterol biosynthesis, FEBS Lett. 580 (2006) 5442–5449.
[4] L. Goedeke, C. Fernández-Hernando, Regulation of cholesterol homeostasis, Cell. Mol. Life Sci. (2012) 1–16.
[5] M.S. Brown, J.L. Goldstein, Multivalent feedback regulation of HMG CoA reductase, a control mechanism coordinating isoprenoid synthesis and cell growth, J. Lipid Res. 21 (1980) 505–517.
[6] J.L. Goldstein, M.S. Brown, et al., Regulation of the mevalonate pathway, Nature 343 (1990) 425.
[7] J.W. Porter, S.L. Spurgeon, Biosynthesis of Isoprenoid Compounds, Wiley, New York, 1981.
[8] Z.H. Beg, J.A. Stonik, H.B. Brewer, 3-Hydroxy-3-methylglutaryl coenzyme A reductase: regulation of enzymatic activity by phosphorylation and dephosphorylation, Proc. Natl. Acad. Sci. 75 (1978) 3678–3682.
[9] M.S. Brown, S.E. Dana, J.L. Goldstein, Regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in human fibroblasts by lipoproteins, Proc. Natl. Acad. Sci. 70 (1973) 2162–2166.
[10] K. Gibson, G. Hoffmann, A. Schwall, R. Broock, S. Aramaki, L. Sweetman, et al., 3-Hydroxy-3-methylglutaryl coenzyme A reductase activity in cultured fibroblasts from patients with mevalonate kinase deficiency: differential response to lipid supplied by fetal bovine serum in tissue culture medium, J. Lipid Res. 31 (1990) 515–521.
[11] G. Popjak, G. Boehm, T.S. Parker, J. Edmond, P.A. Edwards, A.M. Fogelman, Determination of mevalonate in blood plasma in man and rat. Mevalonate "tolerance" tests in man, J. Lipid Res. 20 (1979) 716–728.
[12] D.J. Shapiro, J.L. Nordstrom, J.J. Mitschelen, V.W. Rodwell, R.T. Schimke, Micro assay for 3-hdyroxy-3-methylglutaryl-CoA reductase in rat liver and in L-cell fibroblasts, Biochim. Biophys. Acta (BBA)-Enzymol. 370 (1974) 369–377.
[13] M. Hiramatsu, A. Hayashi, H. Hidaka, H. Ueshima, T. Kanno, Enzyme immunoassay of urinary mevalonic acid and its clinical application, Clin. Chem. 44 (1998) 2152–2157.
[14] T. Yoshida, A. Honda, N. Tanaka, Y. Matsuzaki, B. He, T. Osuga, et al., Simultaneous determination of mevalonate and 7α-hydroxycholesterol in human plasma by gas chromatography—mass spectrometry as indices of cholesterol and bile acid biosynthesis, J. Chromatogr. B Biomed. Sci. Appl. 613 (1993) 185–193.
[15] G. Nicolau, S. Shefer, G. Salen, E. Mosbach, Determination of hepatic 3-hydroxy-3-methylglutaryl CoA reductase activity in man, J. Lipid Res. 15 (1974) 94–98.
[16] J. Waldron, C. Webster, Liquid chromatography–tandem mass spectrometry method for the measurement of serum mevalonic acid: a novel marker of hydroxymethylglutaryl coenzyme A reductase inhibition by statins, Ann. Clin. Biochem. 48 (2011) 223–232.
[17] A. Honda, Y. Mizokami, Y. Matsuzaki, T. Ikegami, M. Doy, H. Miyazaki, Highly sensitive assay of HMG-CoA reductase activity by LC–ESI–MS/MS, J. Lipid Res. 48 (2007) 1212–1220.
[18] E. Kindt, G. Szekely-Klepser, S.T. Fountain, The validation of a simple LC/MS/MS method for determining the level of mevalonic acid in human plasma, Biomed. Chromatogr. 25 (2011) 323–329.
[19] J. Kuhn, C. Götting, K. Kleesiek, et al., Simultaneous measurement of amiodarone and desethylamiodarone in human plasma and serum by stable isotope dilution liquid chromatography–tandem mass spectrometry assay, J. Pharm. Biomed. Anal. 51 (2010) 210.
[20] P.M. Bossuyt, J.B. Reitsma, D.E. Bruns, C.A. Gatsonis, P.P. Glasziou, L.M. Irwig, et al., The STARD statement for reporting studies of diagnostic accuracy: explanation and elaboration, Ann. Intern. Med. 138 (2003) W1–12.
[21] T. Yoshida, A. Honda, H. Miyazaki, Y. Matsuzaki, Determination of key intermediates in cholesterol and bile acid biosynthesis by stable isotope dilution mass spectrometry, Anal. Chem. Insights 3 (2008) 45.
[22] G. Saini, T. Wani, A. Gautam, B. Varshney, T. Ahmed, K. Rajan, et al., Validation of the LC–MS/MS method for the quantification of mevalonic acid in human plasma and determination of the matrix effect, J. Lipid Res. 47 (2006) 2340–2345.
[23] M.Y. Ndong-Akoume, D. Mignault, S. Perwaiz, G.L. Plaa, I.M. Yousef, Simultaneous evaluation of HMG-CoA reductase and cholesterol 7α-hydroxylase activities by electrospray tandem MS, Lipids 37 (2002) 1101–1107.
[24] S.M. Houten, M.S. Schneiders, R.J.A. Wanders, H.R. Waterham, Regulation of isoprenoid/cholesterol biosynthesis in cells from mevalonate kinase-deficient patients, J. Biol. Chem. 278 (2003) 5736–5743.