We have developed a simple, precise, and ultra-sensitive enzymatic method for measuring serum mevalonic acid (MVA) concentration, which is thought to be a good indicator of the in vivo cholesterol biosynthesis rate. This assay is based on an enzyme cycling reaction and makes use of HMG-CoA reductase (HMGR), thio-NAD, NADH, and CoA. MVA participates in the HMGR cycling reaction, and its level is measured based on the production of thio-NADH, which is determined from the change in absorbance at 405 nm. To achieve high specificity, we used mevalonate kinase (MVK) in addition to HMGR. Only substrates able to participate in both the HMGR cycling reaction and the MVK reaction are measured as MVA. The detection limit for MVA is 0.4 ng/ml (2.7 nmol/l), and the calibration curve for MVA is linear up to 44 ng/ml (300 nmol/l). Regression analysis with 40 serum samples showed the accuracy of quantifying MVA with this enzymatic assay to be comparable to that using LC-MS/MS (correlation: $y = 0.83x + 0.24$; $r = 0.97$). This procedure is simple, precise, and robust. It is also rapid and has a high throughput, making it potentially useful for clinical applications.

Cholesterol originates from dietary intake and from de novo synthesis. HMG-CoA reductase (HMGR) has long been established as the rate-limiting enzyme in the cholesterol biosynthetic pathway (1), and the level of mevalonic acid (MVA), the product of HMGR, in plasma appears to be a good indicator of de novo cholesterol synthesis (1). Two main types of drugs are in use to lower serum cholesterol: HMGR inhibitors and intestinal cholesterol absorption inhibitors. HMGR inhibitors, also called statins, include pravastatin, simvastatin, and atorvastatin; these compounds lower cholesterol mainly by inhibiting cholesterol synthesis. On the other hand, ezetimibe inhibits the intestinal absorption of cholesterol in the diet. Targeting the appropriate therapy for individual patients could potentially be improved by understanding the underlying causes of hypercholesterolemia by monitoring the levels of plasma and/or urinary MVA. Such measurements would also be valuable in monitoring the response to treatment.

A number of methods for measuring serum, plasma, or urinary MVA have been reported. These include a radioenzyme assay (2), enzyme immunoassay (3), GC-MS assay (4–7), and LC-MS/MS assay (8–13). Until now, however, the use of enzymatic spectrophotometric methodology has been precluded by the extremely low levels of MVA in human plasma and serum. The normal range of MVA concentration has been reported to be 1.0–11.2 ng/ml (6.7–75.6 nmol/l) in plasma (9) and 12.28 ± 2.54 ng/ml (82.9 ± 17.1 nmol/l) in serum (11). In the present study, we describe a simple, precise, and ultrasensitive enzymatic method for measuring MVA in serum. To achieve high sensitivity to MVA, we used an enzymatic cycling method (14–18) involving the use of HMGR, thionicotinamide adenine dinucleotide (thio-NAD), NADH, and CoA. To achieve high specificity, the HMGR cycling reaction is carried out with and without a preceding mevalonate kinase (MVK) reaction, and the MVA concentration is calculated from the difference between the two results. The MVK reaction eliminates the participation of possible HMGR cycling reaction substrates other than MVA (e.g., HMG-CoA). Using this HMGR cycling method, we determined MVA concentrations in 40 sera and validated the results by comparison with values obtained using LC-MS/MS.

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**Supplementary key words** statin • HMG-CoA reductase • mevalonate kinase • enzyme cycling reaction

**Abbreviations**: HMGl, 3-hydroxy-3-methylglutaryl-CoA lyase; HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; MVA, mevalonic acid; MVK, mevalonate kinase; MVAL, mevalono lactone; thio-NAD, thionicotinamide adenine dinucleotide.

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NADH formation measured as a change in the absorbance of formazan dye at 550 nm. The reaction mixture contained 50 mmol/l Tris-HCl (pH 7.5), 0.005% nitro blue tetrazolium chloride, 5 mmol/l glucose, 1 mmol/l MgCl₂, 1 mmol/l NAD, 1 mmol/l ATP, 5 U/ml ADP-specific glucokinase, 5 U/ml glucose-6-phosphate dehydrogenase, and 2 mmol/l DL-MVAL at 37°C. One unit of enzyme activity was defined as the amount of enzyme forming 1/μmol of NADH per minute under the above conditions.

HMGR enzymatic cycling reaction. HMGR converts MVA and CoA to HMG-CoA with thio-NAD as a cofactor. HMGR then also converts HMG-CoA to MVA and CoA with NADH as a cofactor. This reaction is repeated with accumulation of thio-NADH during the cycling.

**MATERIALS AND METHODS**

**Chemicals**

DL-mevalonolactone (DL-MVAL), DL-HMG-CoA sodium salt trihydrate, fluvastatin sodium hydrate, simvastatin, atorvastatin calcium salt trihydrate, mevinolin (lovastatin), mevastatin, pravastatin sodium salt hydrate, and Proclin 300 were obtained from Sigma-Aldrich (St. Louis, MO). Deuterated MVAL (d7-MVAL) was from CDN Isotopes (Pointe-Claire, Quebec, Canada). ATP, NADH, and thio-NAD were from Oriental Yeast (Tokyo, Japan). Glycine, Tris, ammonium sulfate, nitro blue tetrazolium chloride, CoA, and ascorbic acid were from Wako Pure Chemicals (Osaka, Japan). HEPES, MES, and EDTA were from Dojindo Laboratories (Kumamoto, Japan). “Interference Check A plus” was from Sysmex (Kobe, Japan). Q Sepharose big beads, Phenyl Sepharose fast flow, Q Sepharose fast flow, DEAE-Sepharose fast flow, and Blue Sepharose fast flow were from GE Health Care (Tokyo, Japan). All other chemicals were of the highest quality commercially available.

**Enzymes**

Glucose-6-phosphate dehydrogenase and diaphorase were from TOYOBO (Osaka, Japan). ADP-specific glucokinase and 3-hydroxybutyrate dehydrogenase were from Asahi Kasei Pharma (Tokyo, Japan).

HMGR was purified to homogeneity from overproducing recombinant *Escherichia coli* expressing the HMGR gene from *Pseudomonas putida* KT2440 strain (ATCC47054) as described previously with minor modifications (19). Briefly, the cell lysate was centrifuged, after which the supernatant was subjected to Q Sepharose big beads chromatography, ammonium sulfate precipitation, Phenyl Sepharose fast flow chromatography, Blue Sepharose fast flow chromatography, dialysis, and concentration. The enzyme activity was assayed based on NADH formation measured as a change in the absorbance of formazan dye at 350 nm. The reaction mixture contained 50 mmol/l Tris-HCl (pH 7.5), 0.005% nitro blue tetrazolium chloride, 5 mmol/l glucose, 1 mmol/l MgCl₂, 1 mmol/l NAD, 1 mmol/l ATP, 5 U/ml ADP-specific glucokinase, 5 U/ml glucose-6-phosphate dehydrogenase, 5 U/ml diaphorase, and 2 mmol/l DL-MVAL at 37°C. One unit of enzyme activity was defined as the amount of enzyme forming 1 μmol of NADH per minute under the above conditions.

3-Hydroxy-3-methylglutaryl-CoA lyase (HMGL) was purified to homogeneity from overproducing recombinant *E. coli* expressing the HMGL gene from *Pseudomonas putida* KT2440 strain (ATCC47054) as described previously with minor modifications (19). Briefly, the cell lysate was centrifuged, after which the supernatant was subjected to Q Sepharose big beads chromatography, ammonium sulfate precipitation, Phenyl Sepharose fast flow chromatography, Blue Sepharose fast flow chromatography, dialysis, and concentration. The enzyme activity was assayed based on the oxidation of NADH measured as a decrease in the absorbance at 340 nm. The reaction mixture contained 19.4 mmol/l Tris-HCl (pH 9.5), 0.19 mmol/l NADH, 4.9 U/ml 3-hydroxybutyrate dehydrogenase, and 0.17 mmol/l DL-HMG-CoA at 37°C. One unit of enzyme activity was defined as the amount of enzyme oxidizing 1 μmol of NADH per minute under the above conditions.

**TABLE 1. Limit of detection of MVA in solution**

| MVA (ng/ml) | 0.0 ng/ml | 0.4 ng/ml | 0.6 ng/ml | 0.8 ng/ml | 1.5 ng/ml |
|------------|----------|----------|----------|----------|----------|
| Measured   | 0.06     | 0.41     | 0.59     | 0.79     | 1.58     |
| Average    | 0.00     | 0.40     | 0.57     | 0.81     | 1.63     |
| SD         | 0.05     | 0.07     | 0.02     | 0.05     | 0.12     |
Samples
Serum samples from 40 volunteers were purchased from Kojin Bio (Tokyo, Japan). Pooled plasma and serum were from Kojin Bio (Tokyo, Japan). Control serum, “L-Consera IEX,” was from Nissui Seiyaku (Tokyo, Japan). Solutions of statins were prepared in distilled water (fluvastatin sodium hydrate, simvastatin, atorvastatin calcium salt trihydrate, lovastatin, and mevastatin) or dimethyl sulfoxide (pravastatin sodium salt hydrate) and then added to control serum “L-consera IEX.” Solutions of bilirubin, hemoglobin, and chyle were prepared in “Interference Check A plus” following the manufacturer’s instructions and added to control serum “L-Consera IEX” (1:9 vol).

Preparation of standard MVA solution for the enzymatic method
Because only (R)-MVA (D-MVA) is biologically active in the mevalonate pathway, we prepared a standard MVA solution in which the (R)-MVA concentration was known. The MVA solution was prepared by dissolving DL-MVAL in water and diluting it with water. The (R)-MVA concentration in a racemic MVA solution was determined using the modified HMGR coupled with HMGL method described previously (19). Briefly, all (R)-MVA in the sample was converted, and the amount was calculated based on the increase in NADH.

Preparation of HMG-CoA solution as a calibrator for the enzymatic method
Because the HMGR cycling reaction is run with a preceding MVK reaction, the MVA solution cannot be used as a calibrator because the MVA would be eliminated by the MVK reaction. We therefore adopted HMG-CoA solution as a calibrator, and the levels of the calibrator HMG-CoA were determined using standard (R)-MVA solutions in the HMGR cycling protocol without the MVK reaction, as described below. The HMG-CoA solution was prepared by dissolving DL-HMG-CoA sodium salt trihydrate in water and then diluting with buffer containing 10 mmol/l H2SO4 and 0.1% Tween 80.

Enzymatic measurement of MVA
The enzymatic measurement of MVA is illustrated schematically in Fig. 1. MVA is reversibly converted to HMG-CoA by HMGR, and this cycling reaction is repeated. The MVA concentration is derived from the amount of thio-NADH formed, which is measured based on the change in absorbance at 405/600 nm. The enzymatic assay is illustrated schematically in Fig. 2. Samples are divided into two portions, and one portion is exposed to MVK to convert MVA to phosphomevalonate, which is not to be a substrate for HMGR. 2) The MVK-treated and -untreated samples are then added to the HMGR cycling reaction (the MVK reaction is inactivated by adding EDTA) in which MVA and other possible substrates (e.g., HMG-CoA) are repeatedly oxidized or reduced, causing the accumulation of thio-NADH and NAD. 3) For samples not treated with MVK, the amount of MVA with other possible HMG substrates is measured based on the increase in thio-NADH monitored as an increase in absorbance at 405 nm. With MVK-treated samples, HMG substrates other than MVA are measured. 4) The amount of MVA in the sample is calculated from the difference between the substrate levels in the MVK-treated and -untreated samples.

Measurement of MVA using LC-MS/MS
Measurement of MVA using LC-MS/MS was carried out after conversion of MVA to MVAL, as described previously with slight

### TABLE 2. Within-run precision of MVA spiked in control serum

|   | Pooled serum | MVA solution |
|---|--------------|--------------|
| V  | 14.8 ng/ml   | 14.8 ng/ml   |
| MVA measured (ng/ml) | 3.6 | 18.1 |
| SD | 0.11         | 0.20         |
| CV(%) | 3.7         | 1.1          |
Linearity

Assay linearity was confirmed by making measurements using solutions containing known concentrations of MVA. Linear regression analysis gave the relationship $y = 0.960x + 0.649$ ($r = 1.00$), and the curve was linear to at least 44.4 ng/ml (300 nmol/l).

Lower limit of detection

The lower detection limit of the assay was found to be 0.4 ng/ml by evaluating solutions containing 0, 0.4, 0.6, 0.8, or 1.5 ng/ml MVA with the criterion that the mean ± 3 SD cannot cross zero (Table 1).

Within-run and between-day precision

Within-run precision was assessed by measuring control serum samples, control samples containing 14.8 ng/ml or 7.4 ng/ml exogenous MVA, and a MVA solution containing 14.8 ng MVA/ml. The CVs ranged from 0.8% to 3.1% (Table 2). Between-day precision was assessed by measuring the 7.4 ng/ml MVA solution on three different days using reagent solutions that had been stored at 4°C and calibrated each day. The CV was 0.8%. The enzymatic assay showed good within-run and between-day precision.

Recovery

Known amounts of exogenous MVA were added to pooled serum, pooled plasma, and distilled water and measured using our enzymatic assay. Good recovery was observed in both serum and plasma (Fig. 3).

Interference study by serum substances and statin drugs

The enzymatic assay was not affected by free bilirubin (up to 19.1 mg/dl), conjugated bilirubin (up to 20.0 mg/dl), hemoglobin (up to 489 mg/dl), chyle (up to 144 Formazin Turbidity Units), or ascorbic acid (up to 100 mg/dl) (Table 3). The assay was also not affected by fluvastatin, simvastatin,
The enzymatic method (y) and the LC-MS/MS (x) correlation between the two methods was very strong (Fig. 4).

**Correlation between the enzymatic assay and LC-MS/MS**

When MVA levels were measured in 40 individual serum samples using our enzymatic assay and LC-MS/MS, the correlation between the two methods was very strong (Fig. 4).

**DISCUSSION**

Our HMGR enzyme cycling method for assaying MVA has two important and novel features. First, the detection limit of 0.4 ng/ml (2.7 nmol/l) is nearly 100 to 1,000 times lower than the reported limit for the thio-NAD-dependent enzyme cycling method; in fact, this assay has the lowest reported detection limit for any spectrophotometric assay. Second, this is the first enzyme cycling method based on a three-step enzyme reaction. A scheme showing the proposed HMGR-catalyzed reaction between MVA and HMG-CoA is illustrated in Fig. 5 (20). The enzyme cycling reaction occurs between MVA and HMG-CoA, not between MVA and mevaldehyde or between MVA and mevadyl-CoA. As was shown previously (19), the cycling reaction was nearly undetectable without CoA, and adding HMGL to the reaction blocked the cycling reaction completely.

By using MVK, we were able to make the HMGR enzyme cycling reaction highly specific for MVA. Running the reactions in the presence and absence of MVK allows one to subtract out signals produced from substrates other than MVA, such as HMG-CoA, mevaldehyde, mevadyl-CoA, and other possible substrates. However, it necessitates the use of a calibrator other than MVA, which is eliminated by MVK. We used HMG-CoA solution as our calibrator.

Statin drugs did not interfere with the HMGR enzyme cycling assay because there was much more enzyme present in the reaction mixture than is present under physiological conditions and because the affinity of bacterial HMGRs for statins is four orders of magnitude lower than human HMGR (21).

The correlation between the enzymatic assay and LC/MS/MS (Fig. 4) shows that the accuracy of the enzymatic method is comparable to LC-MS/MS, though the sample size was small (n = 40), and LC-MS/MS detects both (R)-MVA and (S)-MVA. Possible reasons for the slope less than 1.0 (0.83) are 1) a small sample size (n = 40) and 2) that LC-MS/MS detects both (R)-MVA and (S)-MVA. Although (S)-MVA is not known to exist in human blood or other biological materials, the presence of D-amino acids and D-lactate raises the possibility that this form could exist and could play an unknown role in one or more processes other than cholesterol biosynthesis. Our present findings address the measurement of MVA only in serum, but this method also appears to be applicable to urine and other tissues. We measured MVA in human urine diluted approximately 1:100 because the MVA concentration is much higher in urine than in serum. Accordingly, this approach may also be applicable to measuring MVA in tissues after extraction and preparation of appropriate samples for the assay.

In conclusion, we have developed a simple, precise, ultrasensitive MVA assay for quantifying MVA in serum without an extraction step. The results of the recovery study suggest the assay is also applicable to plasma. This robust enzyme cycling method is specific, rapid, and capable of high throughput. Its accuracy is comparable to LC-MS/MS, and it can be used with automatic analyzers, which would be useful for clinical application.

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