Quantitation of plasma mevalonic acid using gas chromatography–electron capture mass spectrometry

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Summary Circulating concentrations of mevalonic acid (MVA) change in parallel with, and may be used as a marker of, cholesterol biosynthesis. Plasma MVA levels have been quantified using a sensitive and specific capillary gas chromatography–electron capture mass spectrometric assay. The detection limit for MVA in plasma is 100 pg/ml; the intra-assay variation is 5.11%; the inter-assay variation is 7.7%. Using this assay, the mean plasma MVA in 15 normolipidemic subjects was 2.37 ± 1.2 ng/ml (range 0.41–5.31 ng/ml). Administration of 40 mg of simvastatin (an HMG-CoA reductase inhibitor) significantly accentuated the diurnal decrease in plasma MVA levels. This assay may be useful in investigating cholesterol synthesis rates in different dyslipidemias and individual responses to HMG-CoA reductase-inhibiting drugs. — **Scoppola, A., V. M. G. Maher, G. R. Thompson, N. B. Rendell, and G. W. Taylor.** Quantitation of plasma mevalonic acid using gas chromatography–electron capture mass spectrometry. *J. Lipid Res.* 1991. **32:** 1057–1060.

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Mevalonic acid (MVA) is a normal constituent of rat and human plasma (1). MVA is formed in organs involved in cholesterol synthesis, most notably the liver, by the action of hydroxymethyl glutaryl-coenzyme A (HMG-CoA) reductase and is also a precursor of ubiquinone and dolichol (2). Parker et al. (3, 4) have estimated that about 1% of synthesized MVA spills over into plasma and have shown that plasma concentrations are closely correlated with the rate of cholesterol synthesis in the whole body. These and other authors (5) also noted a circadian variation in plasma levels and urinary MVA excretion in humans. Measurement of MVA concentrations in plasma or urine provides a simpler and more practicable way of assessing human cholesterol synthesis than using conventional sterol balance studies. With the recent introduction of HMG-CoA reductase inhibitor drugs, MVA assays have gained a new clinical significance by providing an additional marker to changes in lipoprotein levels of the efficacy of therapy (6–8).

A number of assays for plasma MVA have been reported. The radioenzymatic technique of Popják and colleagues (9) provides a precise determination of picomole amounts of MVA in plasma ultrafiltrate, but the method has the attendant problems associated with radioisotopes and is complex, requiring the purification of a specific enzyme. Gas chromatography–mass spectrometry (GC–MS) offers, in principle, a more specific and sensitive method for MVA determination. Such a method was first developed by Hagenfeldt and Hellström (1) but yielded only semi-quantitative values for plasma mevalonate in humans. More recently, Del Puppo and colleagues (10) developed an efficient extraction procedure for MVA and applied GC–MS in the electron impact mode to determine plasma MVA levels in normal subjects. We have extended this approach, and have developed a sensitive and specific assay for MVA based on capillary gas chromatography–electron capture mass spectrometry (GC–ECMS). The assay has been used to determine plasma MVA in normolipidemic subjects and the acute effects of an HMG-CoA reductase inhibitor on circulating MVA levels.

METHODS

Materials

Mevalonolactone (MVL), bis(trimethylsilyl)trifluoroacetamide, and diisopropylethylamine were purchased from Sigma (Poole, Dorset, UK). The internal standard 3,5 dihydroxy-3-[$^2H_3$]methyl pentanoic acid 1,5 lactone ($^2H_3$-MVL) was purchased from MSD Isotopes (Montreal, Canada). The cation-exchange resin (Dowex 50 H^+ form) was obtained from BDH Chemicals (London, UK), the Lc-Si cartridge from Analytichem International (Luton, Bedfordshire, UK), 3,5-bis(trifluoromethyl)benzyl bromide from Fluka (Fluorochem, Glossop, Derbyshire, UK). Acetonitrile was dried over calcium hydride before use.

Extraction

Mevalonic acid was extracted from 1 ml of plasma using an extension of the method originally described by Del Puppo and colleagues (7), with addition of 10 ng of [$^2H_3$]MVL to each sample as an internal standard. Essentially, plasma was diluted with water (1:1) and incubated with Dowex 50 (H^+) for 1 h to convert free MVA to its lactone. Dowex was removed by filtration and nonpolar lipids were extracted in hexane (1 ml). MVL from the aqueous solution was extracted into dichloromethane–propan-2-ol 9:1(v/v, 3 × 5 ml), which was loaded into a Lc-Si silica cartridge. MVL was eluted in acetone (2 ml), and excess solvent was removed under nitrogen. The lac-

Abbreviations: MVL, mevalonolactone; MVA, mevalonic acid; HMG, 3-hydroxy-3-methylglutaryl; GC–ECMS, gas chromatography–electron capture mass spectrometry; TMS, trimethylsilyl; EIMS, electron impact mass spectrometry.

tone was finally reconverted to free MVA by incubation with 100 μl of di-isopropylethylamine–water 1:3 (v/v) for 1 h at room temperature. Recovery through the extraction procedure was monitored by adding 0.04 μCi of [^{3}H]MVL to samples.

Derivatization

Mevalonic acid was converted to its 3,5-bis(trifluoromethyl)benzyl ester by addition of acetonitrile (30 μl), di-isopropylethylamine (10 μl), and 3,5-bis(trifluoromethyl)benzyl bromide (10 μl). After 30 min at room temperature the reagents were removed under a stream of nitrogen. The trimethylsilyl ether (TMS) derivative was then prepared by incubation with 30 μl of bis(trimethylsilyl)trifluoroacetamide for 18 h at room temperature or at 60°C for 1 h. Excess reagent was removed under nitrogen, and the samples were reconstituted in octane (10 μl) for GC–MS.

Gas chromatography–mass spectrometry

Derivatives were chromatographed on a DB5 capillary GC column (30 m, Jones Chromatography) using helium as the carrier gas. The column was held at 100°C for 1 min, followed by a linear temperature gradient of 20°C/min to 325°C. A Grob injector was used in the splitless mode at 250°C. The GC column was routed into the ion source of a Finnigan 4500 quadrupole mass spectrometer operated under electron capture conditions. Ammonia was used as a source of thermal electrons (P approx. 0.4 torr, 100 eV electron energy). The mass spectrometer (MS) was operated in the selected ion mode monitoring ions at *m/z* 291 and *m/z* 294 for the detection of derivatized MVA and [$^2H_3$]MVA, respectively. Quantitation of MVA was carried out by determining the peak area ratios (*m/z* 291/294) and comparing them against an extracted standard curve (0.25–20 ng of MVA).

Subjects

Fifteen control subjects, 8 male and 7 female, aged 18–57 years were studied. All were normolipidemic; their fasting serum total cholesterol and triglyceride values ranged from 3.6 to 5.4 and 0.25 to 0.99 mmol/l, respectively. Blood samples (10 ml) for MVA assay were heparinized, and the plasma was separated by centrifugation and then stored at −20°C. Samples were taken at 9 AM after a 12-h fast. In 8 subjects samples were also taken at 10 AM, 12 PM, 2 PM, and 4 PM on two separate days, on one of which they received a single dose of simvastatin, 40 mg, at 10 AM. Low fat (<5%), cholesterol-free meals were eaten at 9:30 AM and 12:30 PM on both occasions.

RESULTS

Mevalonic acid is readily converted to the di-(O-trimethylsilyl)-bis(trifluoromethyl)benzyl derivative under mild conditions. The derivative chromatographs well on the GC column eluting with a retention time of 5.3 min. Under electron capture conditions, this derivative generates a mass spectrum (shown in **Fig. 1a**) where fragment ion $(M\text{-}(CF_3)_2 \cdot C_6H_3 \cdot CH_2)^-$ at *m/z* 291 is the base peak and there is very little further fragmentation. A commercially available deuterated analogue, [$^2H_3$]MVA (used as internal standard in the assay), elutes from the gas chromatography column 5 sec earlier; it generates a

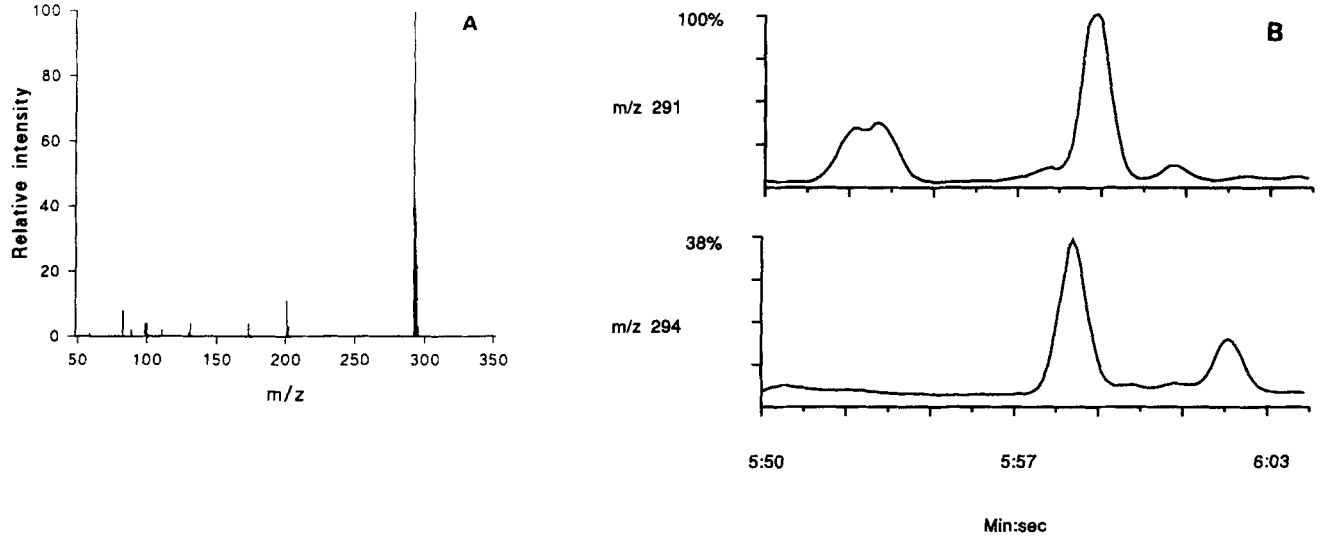

Fig. 1. A: Electron capture mass spectrum of the di-(O-trimethylsilyl)-bis(trifluoromethyl)benzyl derivative of MVA. The base peak in the spectrum is the fragment $(M\text{-}(CF_3)_2 \cdot C_6H_3 \cdot CH_2)^-$ at *m/z* 291. B: Representative ion profile for *m/z* 291 (sample) and *m/z* 294 ([$^2H_3$]MVA, 10 ng internal standard) of extracted plasma.

TABLE 1. Data on plasma mevalonate values in fasted humans

Subjects	n	Mean	Range (Observed or SD)	Method of Assay	Source (Reference)
		ng/ml			
Unspecified	8	3.04	± 0.35	Radioenzymatic	Popják et al. (9)
Unspecified	14	4.98	± 0.76	Radioenzymatic	Popják et al. (9)
Unspecified	4	8.85	± 0.91	Radioenzymatic	Popják et al. (9)
Controls	40	5.59	1.56–13.3	Radioenzymatic	Parker et al. (3)
Controls	5	16.3	12.4–18.2	Radioenzymatic	Illingworth et al. (6)
FH[a]	4	27.3	22.8–32.5	Radioenzymatic	Illingworth et al. (6)
IIa and IV[b]	16	10.5	± 2.4	GC-MS	Del Puppo et al. (10)
Controls	15	2.37	± 1.2	GC-MS	This study

Factor used to convert pmol/ml to ng/ml = × 0.13.
[a]Familial hypercholesterolemia.
[b]Type IIa or IV hyperlipidemia.

similar mass spectrum but with the major ion shifted by 3 u to *m/z* 294.

With our modified method, recovery of free mevalonic acid was always > 76% as determined by inclusion of a radiolabeled tracer in some experiments. After extraction from plasma, the GC baseline for ion channels *m/z* 291 (MVA) and 294 ([$^2H_3$]MVA) was remarkably clean; there was little difficulty identifying the peaks of interest (Fig. 1b). This is in contrast to the EIMS method of Del Puppo and colleagues (10), which in our hands resulted in a much poorer ion profile. Although less than 10 pg of derivatized MVA could be detected by this method, the true limit of detection in plasma was 110 pg/ml based on extracted standards. The coefficients of variation were 5.12% within assays and 7.7% between assays. MVA concentrations in urine were determined as easily as in plasma (data not shown).

Plasma MVA concentrations at 9 AM in 15 normal subjects averaged 2.37 ± 1.20 ng/ml (SD), with values ranging from 0.41 to 5.31 ng/ml. These values tend to be somewhat lower than those observed by some other authors, as shown **Table 1**. It remains to be determined whether these differences represent biological or methodological variation.

The diurnal variation in MVA in a normal subject is shown in **Fig. 2**. Acceleration of the time to reach the nadir of plasma MVA (at 4 PM) and accentuation of its depth by a single dose of simvastatin in 8 normal subjects is illustrated in **Fig. 3**.

DISCUSSION

The introduction of a new class of cholesterol-lowering drugs, the HMG-CoA reductase inhibitors, has revived interest in determining plasma MVA levels as a marker of endogenous HMG-CoA reductase activity. Using the original GC-MS method of Del Puppo and colleagues (10), we found that the GC baseline was markedly variable, with, in some instances, a number of impurities interfering with the quantitation of MVA. This was partly due to the low mass of the ion (*m/z* 145) used for quantitation in

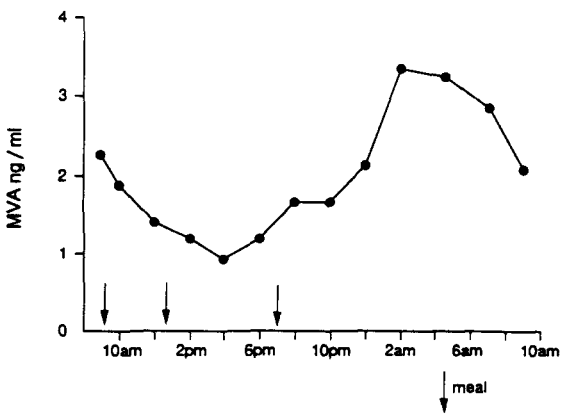

Fig. 2. Diurnal variation in plasma MVA in a normal subject (less than 5% dietary fat). Meal times are indicated by arrows. Peak and trough values occurred at 2 AM and 4 PM, respectively.

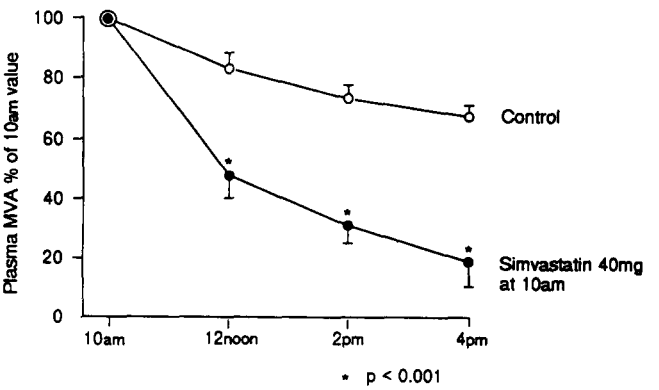

Fig. 3. Effect of simvastatin (an HMG-CoA reductase inhibitor) on diurnal change in plasma MVA in eight normal subjects. Subjects were fasted overnight until 9 AM when they received a low fat, cholesterol-free breakfast. Simvastatin, 40 mg, was administered immediately after the 10 AM blood sample had been taken. Mean (± SEM) percentage changes from baseline values (10 AM) are shown. Values were significantly lower at 12 noon, 2 PM, and 4 PM ($P < 0.001$).

this assay. In their original extraction protocol, these authors found that the lactone (MVL) was more readily extracted and purified than the free acid (MVA). Thus, plasma was acidified and the MVA was extracted as the lactone (MVL). The lactone, unfortunately, possesses only a single free hydroxyl group available for derivatization, resulting in a low molecular weight derivative. If, however, the lactone is hydrolyzed subsequent to extraction, the free dihydroxy acid (MVA) is readily converted, under very mild conditions, to a species of higher molecular weight, the di-(O-trimethylsilyl)-bis(trifluoromethyl) benzyl ester.

The problem of co-eluting impurities affecting the baseline was exacerbated by the relatively high limit of detection of this EIMS-based assay, MVA in some plasma samples being below measurable values. Under EI conditions, the derivative extensively fragments in the mass spectrometer, with only a small proportion of the total ion current carried by any one ion. With electron capture ionization, however, fragmentation is minimal and detection limits are much lower. The assay reported here makes use of the electron-capturing bis(trifluoromethyl)benzyl derivative; this derivative, together with its benzoyl analogue, is finding increasing application in GC-ECMS assays (11-12). The GC-ECMS method outlined in this report gives increased specificity and, to some extent, increased sensitivity over previous methods for MVA analysis. Using this assay, circulating levels of MVA in normolipidemic human subjects have been determined, the diurnal rhythm of MVA has been confirmed, and the acute effects of an HMG-CoA reductase inhibitor have been demonstrated. This assay should prove useful in a number of areas where a knowledge of cholesterol biosynthesis is required and could be used, for example, to compare cholesterol synthesis rates in different types of dyslipidemia.

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