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A sensitive and selective method for the determination of mevalonic acid in dog plasma by gas chromatography/negative ion chemical ionization-mass spectrometry[1]

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Abstract

A sensitive and selective method has been developed for the determination of mevalonic acid (MVA), a cholesterol biosynthetic precursor, in dog plasma using solid-phase extraction in combination with gas chromatography/negative ion chemical ionization-mass spectrometry (GC/NICI-MS). MVA extracted from plasma with a phenylboronic acid-bonded phase cartridge was converted to its pentafluorobenzyl (PFB) ester-cyclic boronate derivative to produce a carboxyltate anion $[M\text{-}PFB]^-$ in the NICI mode. PFB ester boronate derivatives of MVA and its internal standard, d_3-mevalonolactone, were monitored in the selected ion mode at m/z 213 and 216, respectively. The precision and accuracy of within-run and between-run assays were within 8%. This method was used to follow the diurnal variation of MVA levels in plasma of fasted and fed dogs. The diurnal variations of plasma MVA levels observed between the two groups were similar to those reported previously for human and rat plasma. © 1997 Elsevier Science B.V.

Keywords: Gas chromatography/negative ion chemical ionization-mass spectrometry; Mevalonic acid; Dog plasma; Solid-phase extraction; Phenylboronic acid-bonded cartridge; Pentafluorobenzyl ester; Cyclic boronation; Diurnal variation

1. Introduction

Mevalonic acid (MVA) is an important intermediate in cholesterol biosynthesis, which is regulated by 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase. Previous studies have shown that the plasma concentration and urinary excretion of MVA reflect short- and long-term changes of cholesterol biosynthesis in animals [1] and humans [1–3]. Thus, accurate determinations of MVA in such body fluids can be used to monitor biochemical responses. MVA levels in human plasma and urine were reported to be 0.5–10 ng ml^{-1} [1–5] and 50–300 ng ml^{-1} [6,7], respectively. Thus, a more sensitive and selective method is needed to assay plasma samples. One very sensitive method is the radioenzymatic assay

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developed by Popják et al. [1], but it requires a complicated and time-consuming procedure.

Several gas chromatography/mass spectrometric (GC/MS) methods have been proposed for the development of more specific assays of MVA in biological fluids, following the first report in 1972 by Hangenfeldt and Hellström [8]. These methods use highly toxic solvents such as dichloromethane [5,9–11], chloroform [11,12], and benzene [11] to extract mevalonolactone (MVL), which is less polar than MVA, from aqueous body fluids or from various solid phases for final detection or for pretreatment prior to the following derivatization. They also may require careful evaporation to prevent loss of MVL [12]. Some of these methods have other disadvantages, such as a lack of sensitivity which makes it unsuitable for trace analysis of plasma MVA [8,9,12] and the need for high-resolution MS which requires a high-grade instrument and a skilled operator [11].

The present investigation was undertaken to develop a new GC/MS method to overcome the disadvantages of previous methods. First, our attention was directed to the 3,5-diol moiety of MVA, which was expected to interact with boronic acids. This interaction was used for selective solid-phase extraction of MVA on a phenylboronic acid (PBA)-bonded column to yield the first pretreatment method without a lactonization process, and for the derivatization with *n*-butylboronic acid, which is more selective than a method using silylation agents [5,11]. Second, the carboxyl moiety of MVA was utilized for derivatization with pentafluorobenzylbromide (PFB-Br). The PFB ester derivative was expected to yield a strong characteristic carboxylate anion fragment under negative ion chemical ionization (NICI)-MS conditions [13]. These efforts led to a sensitive and selective method for the determination of MVA in dog plasma by GC/NICI-MS. Our new method was then used to follow the circulating MVA levels in plasma of fasted and fed dogs.

2. Experimental

2.1. Materials

MVA was prepared as a dibenzylethylenediammonium salt in our laboratories according to the previous paper [14] using dibenzylethylenediamine purchased from Tokyo Kasei Kogyo (Tokyo, Japan). The internal standard (IS), d_3-MVL, was purchased from MSD Isotopes (Montreal, Canada). PFB-Br, *N*,*N*-diisopropylethylamine, and *n*-butylboronic acid were from Tokyo Kasei Kogyo (Tokyo, Japan), and quinuclidine from Aldrich (Milwaukee, WI, USA). An HMG-CoA reductase inhibitor, monocalcium bis(+)-7-[4-(4-fluorophenyl)-6-iospropyl-2-(*N*-methyl-*N*-methanesulfonylaminopyrimidin)-5-yl]-(3R,5S)-dihydroxy-(E)-6-heptenoate (S-4522) was synthesized in our laboratories [15]. Bond Elut C18, PBA, and CN cartridges were purchased from Varian (Harbor City, CA, USA). All solvents were of analytical grade. Ethyl acetate was dried over 4 Å molecular sieves prior to use.

2.2. Extraction of MVA from dog plasma

To 1 ml of dog plasma, 50 μl of d_3-MVL MeOH solution (400 ng ml^{-1}) was added and the plasma was deproteinized by addition of 3 ml of MeOH. The mixture was shaken vigorously for 10 min and then centrifuged at 2500 rpm ($970 \times g$) for 10 min. Next, 3 ml of supernatant was evaporated to dryness at 50°C using nitrogen after addition of 1 ml of 0.01 M quinuclidine. The residue dissolved in 2.5 ml of distilled water was loaded onto a Bond Elut C18-PBA joint cartridge, which was composed of a C18 cartridge (100 mg/1 ml) prewashed with 2 ml of distilled water, and a PBA cartridge (100 mg/10 ml) prewashed successively with 5 ml of MeCN and 2 ml of distilled water. After MVA had become adsorbed on the PBA cartridge through the upper C18 cartridge under reduced pressure, the C18 cartridge was removed and the remaining PBA cartridge was washed with 2 ml of MeCN. MVA was eluted with 10 ml of 0.01 M quinuclidine in MeOH and the eluate was evaporated to dryness at 50°C using nitrogen.

2.3. Derivatization

To MVA extracted from dog plasma, 50 μl of 5% PFB-Br in dimethylformamide (DMF) and 10 μl of *N*,*N*-diisopropylethylamine were added. The mixture was allowed to stand for 20 min at room temperature to convert MVA to its PFB ester (MVA-PFB ester). The reaction mixture was evaporated to dryness at room temperature using nitrogen. The residue dissolved in 0.5 ml of a mixed solvent of *n*-hexane-AcOEt (3:2, v/v) was loaded into a Bond Elut CN (500 mg/3 ml) cartridge prewashed successively with 4 ml each of AcOEt and *n*-hexane. The CN cartridge was washed with 4 ml of *n*-hexane-AcOEt (10:1, v/v). The MVA-PFB ester was eluted with 4 ml of *n*-hexane-AcOEt (1:1, v/v). After addition of 25 μl of 0.02% *n*-butylboronic acid in AcOEt, the eluate was evaporated to dryness under reduced pressure at 5°C to convert the MVA-PFB ester to its boronate derivative. After the residue was dissolved in 200 μl of AcOEt, a 2 μl portion of the solution was injected into the following GC/MS system.

2.4. Gas chromatography/mass spectrometry

GC/MS was conducted on a Varian MAT 44S mass spectrometer coupled to a Varian Model 3700 gas chromatograph (Bremen, FRG) with a moving needle injector and a capillary column, PM-65HT (30 m × 0.32 mm ID, 0.1 μm, Quadrex, USA). Operating temperatures were as follows: injector, 300°C; column oven 155°C; transfer line, 150°C; ion source, 145°C. Helium was used as the carrier gas, with an inlet pressure at 25 psi, and 2-methylpropane was used as the reagent gas. The mass spectrometer was operated in the selected ion mode monitoring both ions at *m*/*z* 213 and 216 for the detection of the PFB ester boronate derivatives of MVA and IS, respectively.

2.5. Calibration curve

MVA and d_3-MVL were dissolved with MeOH to prepare stock solutions of 0, 40, 100, 200 and 400 ng ml^{-1} of MVA containing 400 ng ml^{-1} of d_3-MVL. Plasma standard solutions were prepared in duplicate at each concentration by adding 50 μl each of the stock solutions to 1 ml of dog control plasma. The calibration curve was constructed by plotting the peak intensity ratio (y) of the PFB ester boronate derivative of MVA to that of IS against the spiked analyte concentration (x), and by determining the best fit line ($y = bx + a$) from the two-stage weighted least squares regression analysis by the method of Aarons et al. [16]. Actual MVA concentrations (X) were calculated using the line slope (b) and the response ratio (Y), according to the following equation, $X = Yb^{-1}$.

2.6. Animals

Male ($n = 3$) and female ($n = 3$) beagle dogs, approximately 8 months old, weighing 9–11 kg were obtained from CSK Research Park (Nagano, Japan). The animals were made to fast for 23 h until 9.00 am then assigned to two groups, one fed and the other subject to fasting, for assay of the diurnal MVA levels in plasma in a cross-over experiment after an interval of 4 days. The feeding-group animals were fed between 9 and 10 am, while the fasting-group animals received no food. After this, all the animals were not given food until 10 am the next morning.

2.7. Plasma sample

Diurnal level plasma samples separated from heparinized blood by centrifugation were collected at 10 and 11 am, 12 noon, 2 and 4 pm, and 10 am the next morning, and stored at −20°C until analysis. The control plasma samples were pooled after being drawn from the dogs 1 week before the above diurnal level testing. S-4522-treated plasma samples were collected at 2 and 4 h, following single oral administration of 1 or 3 mg kg^{-1} of S-4522 to the dogs under the fasting condition at 10 am, 5 days after the diurnal testing.

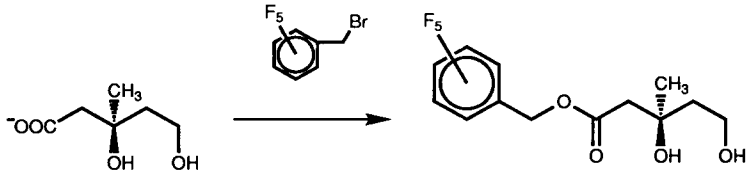

Fig. 1. PFB esterfication of MVA with pentafluorobenzylbromide.

3. Results and discussion

3.1. Derivatization

A new two-step derivatization method for detection of MVA levels in an NICI mode was investigated. The first step of derivatization was PFB esterification of the carboxyl moiety of MVA with PFB-Br in the presence of *N,N*-diisopropylethylamine (Fig. 1). The introduction of a PFB group into the carboxyl function [13] of MVA would be very favorable for the formation of a characteristic carboxylate anion in the NICI mode. First, the reaction of MVA with PFB-Br in MeCN, which had been frequently used as a benzyl esterification solvent [13,17], was tried. However, there was an undesirable side reaction of decomposition of the produced MVA-PFB ester to MVL. When DMF solvent was used, MVA showed satisfactory PFB-esterification without MVL production. The effects of PFB-Br concentration and *N,N*-diisopropylethylamine amount on MVA derivatization were examined at room temperature. The maximal levels of MVA-PFB ester formation were obtained in the ranges of 0.5–5% (v/v) PFB-Br and 1–20 μl of the amine. The time course for PFB esterification of MVA was examined at room temperature. As shown in Fig. 2, the PFB ester reached a maximal level in 20 min. Attempts to remove the excess PFB-Br, remaining after PFB esterification, with a silica- or NH_2-bonded phase cartridge resulted in decomposition of the produced MVA-PFB ester to MVL. Finally, the excess PFB-Br was successfully removed without decomposition using normal phase chromatography with a CN-bonded phase cartridge.

As the second step of the derivatization, cyclic boronation of the diol group of the MVA-PFB ester with *n*-butylboronic acid was examined (Fig. 3). The derivatization with boronic acids that specifically binds 1,2- or 1,3-diol group [18] was expected to facilitate the selective determination of MVA. As shown in Fig. 4, the cyclic boronation in AcOEt proceeded rapidly in 10 min at room temperature and 60°C without significant increase of reaction yield by further time consumption, and the yield was increased by evaporation of the solvent after the reaction. Furthermore, the derivatization was found to proceed during the evaporation process of the reaction mixture of the PFB ester fraction eluted from the CN cartridge, and *n*-butylboronic acid. It is probable that the evaporation promoted the reaction by concentration of the mixed solution and/or by removal of the water produced by dehydration. However, the yield of volatile MVA-PFB boronate derivative fluctuated, due to its volatility during evaporation using nitrogen. In order to prevent loss of the derivative produced, the reaction mixture was evaporated to dryness under reduced pressure at 5°C. A nearly constant yield of the derivative was obtained in the range

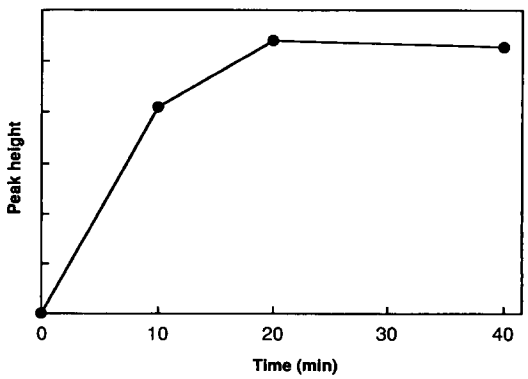

Fig. 2. Time course for PFB esterification of MVA.

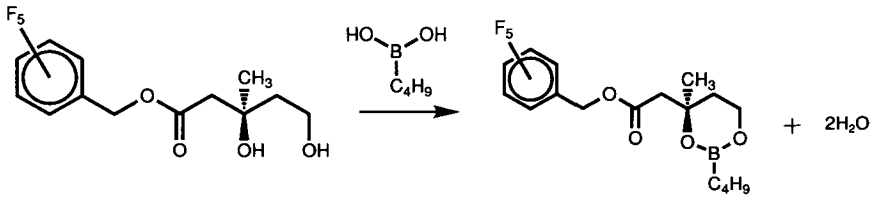

Fig. 3. Cyclic boronation of MVA-PFB ester with *n*-butylboronic acid.

of 2.5–100 μg per tube of *n*-butylboronic acid. A small amount 5 μg per tube of *n*-butylboronic acid was preferable to minimize adsorption of excess reagent on the needle injector of the GC/MS system.

3.2. PBA solid-phase extraction of MVA in plasma

A new method for solid-phase extraction of MVA using a PBA-bonded phase cartridge which had been known to have a high affinity for compounds containing the *cis*-diol group [19] was investigated, as the 3,5-diol moiety of MVA was expected to interact with the cartridge. First, the adsorption and elution behavior of MVA on the cartridge was tested under plasma-free conditions MVA applied to the cartridge under neutral condition was strongly adsorbed without being eluted with H_2O, MeOH, MeCN, and their mixtures within 3 ml. The ability to elute MVA was examined using aqueous MeOH solution containing 0.01 and 0.1 M volatile acids or bases (Fig. 5), because non-volatile acids or bases were difficult to remove after elution and interfered with the following derivatizations. Acidic eluents, which had been expected to yield high elution recovery [19], failed to sufficiently elute the strongly adsorbed MVA. In contrast, basic quinuclidine MeOH solution gave good elution recoveries of more than 80%. Similar elution behavior has been reported for a stable trigonal boronate complex of thromboxane B_2- or 2,3-dinor-thromboxane B_2-methoxime derivative containing the *β*-diol group which was formed on a PBA-bonded phase cartridge and then eluted with alkaline MeOH [20]. A six-membered ring similar to that of the methoxime derivative-PBA complex [20] may be formed between the 3,5-diol group of MVA and PBA. However, there may be other complex forms, because of the presence of the carboxyl

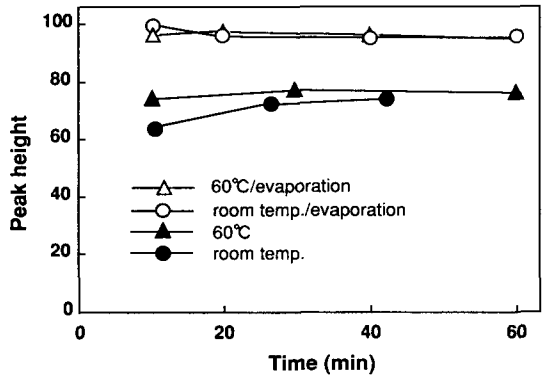

Fig. 4. Time course for cyclic boronation of MVA-PFB ester. MVA-PFB ester purified with CN cartridge was allowed to react with 5 μg of *n*-butylboronic acid in 100 μl of AcOEt at room temperature (●, ○) or 60°C (▲, △). (●, ▲): the reaction solutions were directly subjected to analysis; (○, △): the reaction solutions were evaporated under reduced pressure at 5°C and then the residues dissolved in 100 μl of AcOEt were subjected to analysis.

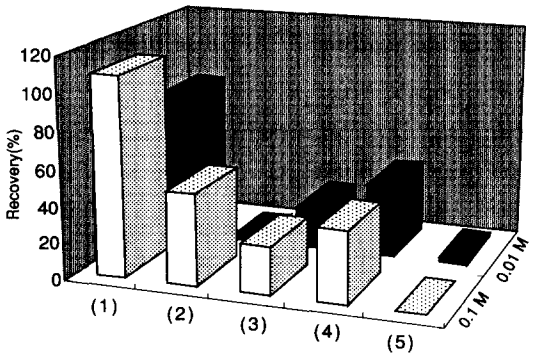

Fig. 5. Effects of acid and base on elution of MVA from PBA-bonded phase. (1) quinclidine, (2) pyridine, (3) ammonia, (4) hydrochloric acid, (5) acetic acid.

group of MVA in addition to the 3,5-diol group of MVA and PBA. As β-hydroxy acids are also known to interact with boronic acids [21], the 1-carboxyl and 3-hydroxyl moieties of MVA may also constitute a six-membered ring complex of the tetrahedral anionic boronate form with PBA. Furthermore, a tridentate complex similar to that of triol condensed with boronic acids [22] may be formed by combining the interaction of both the 3,5-diol and β-hydroxy acid groups of MVA with PBA. In all of the above complex formations, the trigonal boronate form of PBA would react with MVA, and tetrahedral anionic PBA under alkaline conditions would interfere with the above reactions. This is likely to be responsible for the high elution recovery of MVA from the PBA cartridge with the quinuclidine MeOH solvent. Volatile quinuclindine caused no interference of the following PFB esterification.

Because of slight adsorption of MVA in the plasma matrix on the PBA-bonded phase, the deproteinized plasma sample was applied to a C18-PBA joint cartridge, in which a C18-bonded phase cartridge was used with the hope of removing non-polar plasma components. As the result, MVA could be successfully bound to the PBA bonded phase. After the C18 cartridge was removed, the remaining PBA cartridge was washed with 2 ml of MeCN. Recovery of MVA from the PBA cartridge was about 70% using 0.01 M quinuclidine/MeOH as the eluent.

3.3. GC/MS analysis of MVA in plasma

In the NICI mode using 2-methylpropane as the reagent gas, the MVA-PFB ester boronate derivative exhibited its characteristic negative ion $[M\text{-}181]^-$ formed by elimination of the PFB group as shown in Fig. 6. A typical chromatogram of selected ion recording for analysis of MVA extracted from dog plasma is presented in Fig. 7. As shown by the parameters for a typical calibration curve in Table 1, the analytical responses were linear for the dog plasma spiked with 0–20 ng ml^{-1} of MVA.

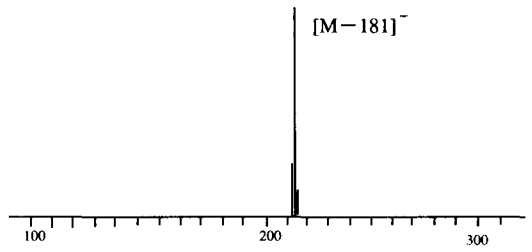

Fig. 6. NICI mass spectrum of MVA-PFB boronate derivative.

3.4. Method validation

A fully validated analytical method needs to be used for reliable determinations of MVA in diurnal variation levels and also the decreases caused by treatment with HMG-CoA reductase inhibitors. Actual MVA levels were calculated using the slope of the calibration curve obtained from the plasma standards which were prepared by adding MVA to dog plasma. The slope was compared with that of a linear curve obtained from S-4522-treated plasma spiked with MVA. Both the slopes showed good agreement as shown in Table 1. They were estimated to be equal to the slope of a plasma-free standard curve, which was not used for assay calibration, because its slope may not always match that of plasma standard curve. The use of a matrix standard is ordinarily recommended to eliminate problems such as matrix effects [23].

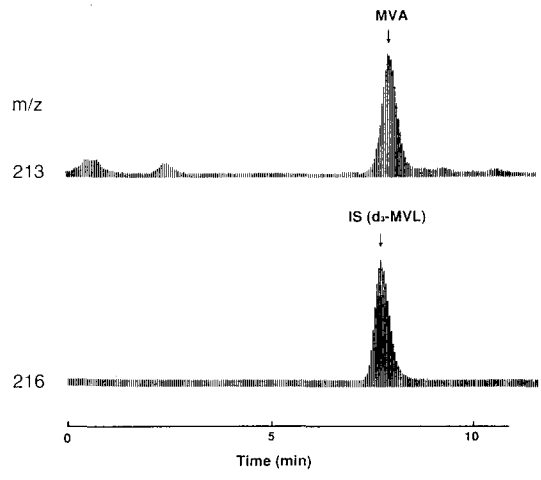

Fig. 7. Chromatograms of the PFB ester boronate derivatives of MVA and IS.

Table 1
Parameters for linear curves obtained from control plasma, S-4522-treated plasma, and plasma-free solution

	Control plasma	S-4522-treated plasma	Plasma-free solution
b	4.51×10^{-2}	4.66×10^{-2}	4.46×10^{-2}
s_b	0.48×10^{-3}	0.30×10^{-3}	0.63×10^{-3}
a	0.302	0.124	0.036
s_a	0.45×10^{-2}	0.74×10^{-2}	0.33×10^{-2}
n	12	12	12

b, Slope; s_b, standard deviation of slope.
a, Intercept; s_a, standard deviation of intercept.

As shown in Table 2, within- and between-run precision and accuracy were assessed to be within 8% by analyzing replicate plasma samples spiked with known amounts of the MVA at concentrations of 0–20 ng ml^{-1}. Analytical performance at a lower MVA concentration was also confirmed to be sufficient as revealed by 3.0% within-run precision for the analysis of S-4522-treated plasma sample.

Table 2
Precision and accuracy for analysis of MVA in dog plasma

	Concentration (ng ml^{-1})			
	Added	Found	R.S.D. (%)	Bias (%)
Within-run ($n = 6$)	0.0	2.7[a]	3.0[a]	—
	0.0	13.5	2.0	—
	5.0	19.5	2.8	+5.4
	10.0	23.9	5.9	+1.7
	20.0	35.9	2.1	+7.2
Between-run ($n = 6$)	0.0	12.4	7.5	—
	5.0	17.4	7.0	0.0
	10.0	22.9	6.1	+2.2
	20.0	33.5	6.9	+3.4

[a]S-4522-treated plasma ($n = 4$).

3.5. Application to analysis of diurnal variation of MVA in dog plasma

Diurnal variation of plasma MVA has been reported in humans [2–4] and rodents [1]. In humans on a normal diet with 3–5 meals/day, the plasma MVA level is maximal at midnight and minimal in the afternoon [2–4]. In the case of a human subject equilibrated on a schedule of one meal (at 7 pm) per day, the plasma MVA was found to vary diurnally with a single peak 12 h after the meal [24]. Changes in dietary conditions, such as the number of and time-zone of meals caused differences in diurnal variation, which was finally abolished by fasting [2,3,24].

Rat plasma MVA was low in the middle or end of a light period and high at the midpoint of a dark period, with the lowest values at the midpoint of the light period in fasted animals [1]. Rats trained to eat during a 4 h period (9 am–1 pm) under normal illumination showed a change in the timing of circadian rhythm of hepatic cholesterol synthesis; its maximum occurred at 6 pm, 9 h after the presentation of food [25]. Similar circadian rhythms have been reported for hepatic cholesterol synthesis in hamsters [26] and HMG-CoA reductase activity in guinea pigs [27].

To the best of our knowledge, the only level reported for MVA in dog plasma is 4.4–16.0 ng ml^{-1} at 12 noon [10]. Our method was applied to the analysis of diurnal variation of MVA in dog plasma under different dietary conditions. Six beagle dogs separated into fasting and feeding groups were tested by cross-over experiment. The initial MVA levels of the two groups at 10 am were almost the same, 10.3 ± 3.3 and 8.1 ± 1.0 (mean ± S.E.), respectively. The MVA levels of the feeding group at 12 noon (9.3 ± 1.1 ng ml^{-1}) were in the range of those reported by Ishihama et al. [10]. As shown in Fig. 8, these levels were 2 times greater than the initial level at 4 pm, or 6 h after food intake, and then returned to the initial level at 10 am the next morning. On the other hand, the fasting group retained the initial MVA levels nearly constantly until the next morning. These observations, the suppression of the diurnal variation in the fasting group and the increase of the MVA level after the ingestion of food, were similar to those reported previously for humans [2] and rats [1].

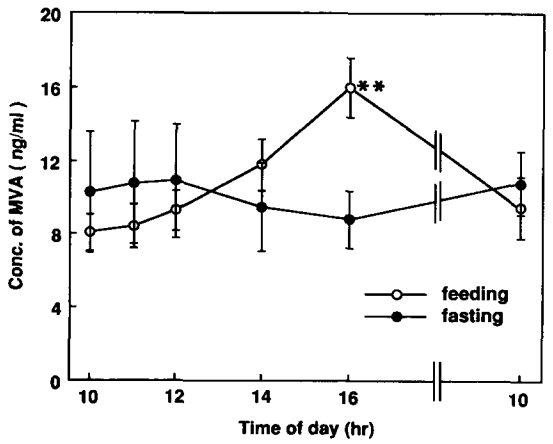

Fig. 8. MVA levels in dog plasma. Values are mean ± S.E. ($n = 6$). $^{**}P < 0.01$.

4. Conclusions

Our GC/NICI-MS assay for MVA is a sensitive, selective, and reliable method which does not require highly toxic solvents. Use of this method yielded data showing that the MVA levels in dog plasma have a diurnal variation pattern similar to those reported previously for humans and rats. This method is expected to be useful for assay of MVA levels in the other biological materials and for follow-up of these levels after administration of various inhibitors of the cholesterol biosynthetic pathway.

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