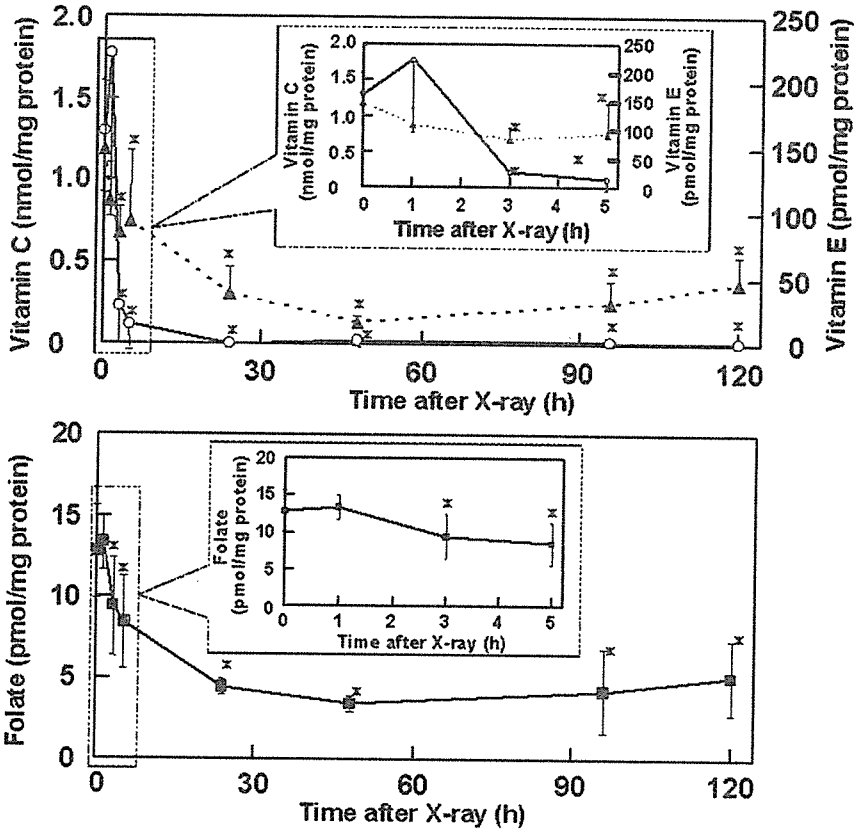

Figure 2. Time-dependent changes in folate, vitamin C and vitamin E in the bone marrow of mice after TBI at 3 Gy. Male ICR mice (4 weeks old) were subjected to TBI via X-rays at a dose of 3 Gy, and then sacrificed at 1, 3, 5, 24, 48, 96, 120 h for the analysis of antioxidant vitamins. Each point (vitamin C, circle; vitamin E, triangle; folate, square) and vertical bar indicates the mean and SD for 5 mice. *Significantly different from non-irradiated level ($p < 0.05$).

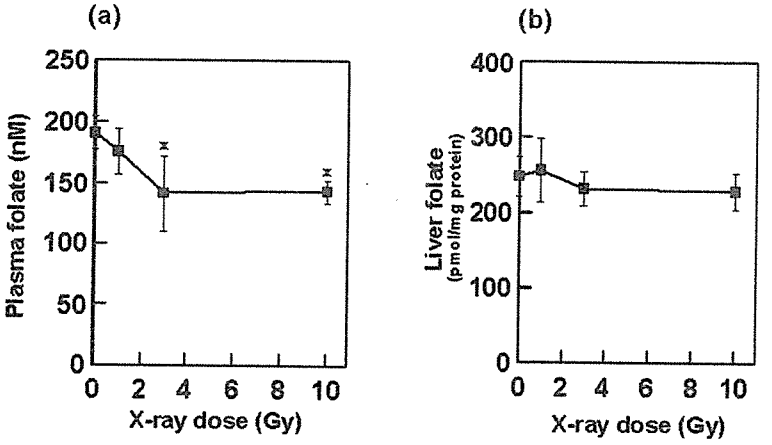

Figure 3. Dose-dependent changes in folate in the plasma (a) and liver (b) of mice after TBI at various doses. Male ICR mice (4 weeks old) were subjected to TBI via X-rays at a dose of 0, 1, 3, 10 Gy. The concentrations of vitamins were determined 24 h after irradiation. Each point and vertical bar indicates the mean and SD for 5 mice. *Significantly different from non-irradiated level ($p < 0.05$).

requirement of folate. In this study, we gave mice TBI, a well-known oxidative stress on the body, and determined the changes of folate status in the plasma, liver and bone marrow in the context of the X-ray dose and post-exposure time. We also compared the changes with those of vitamin C and

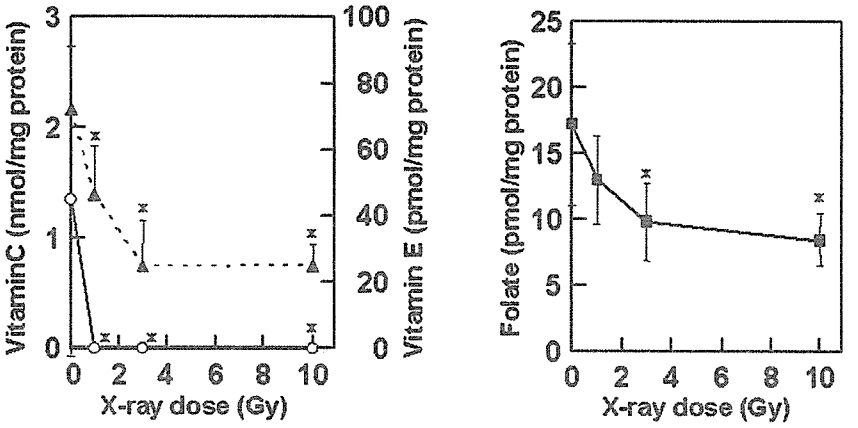

Figure 4. Dose-dependent changes in folate, vitamin C and vitamin E in the bone marrow of mice after TBI at various doses. Male ICR mice (4 weeks old) were subjected to TBI via X-rays at a dose of 0, 1, 3, 10 Gy. The concentrations of vitamins were determined 24 h after irradiation. Each point (vitamin C, circle; vitamin E, triangle; folate, square) and vertical bar indicates the mean and SD for 5 mice. *Significantly different from non-irradiated level ($p < 0.05$).

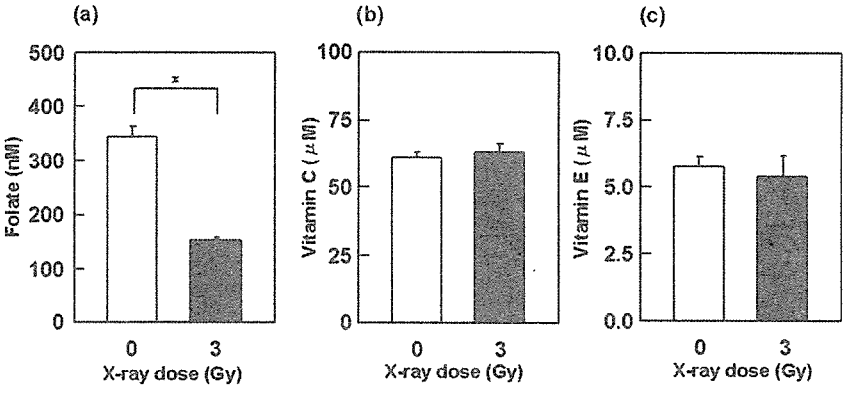

Figure 5. Concentration of folate (a), vitamin C (b) and vitamin E (c) in mice plasma with and without X-ray irradiation *in vitro*. Fresh mice plasma was irradiated with X-ray (3 Gy) and changes in the concentrations of folate, vitamin C and E were measured. *Significant irradiation effect ($p < 0.05$).

vitamin E, which we have reported previously (Umegaki & Ichikawa 1994, Umegaki et al. 1995, 2001).

Bone marrow is particularly susceptible to X-ray irradiation, and marked decreases of vitamin C, vitamin E and folate were detected. The decreases of the three vitamins showed X-ray-dose and exposure-time dependency. Similar to previous studies (Umegaki & Ichikawa 1994, Umegaki et al. 1995, 2001), TBI up to 10 Gy to mice did not decrease vitamin C and vitamin E in the liver and plasma. On the other hand, folate in the plasma was significantly decreased in this study. The results were confirmed by an *in vitro* exposure study (Figure 5). Folate is reported to be degraded by hydroxyl radical and ultraviolet *in vitro* (Off et al. 2005, Patro et al. 2005). The vulnerability of plasma folate by oxidative stress may be related with the high radical scavenging capacity of folate observed in *in vitro* studies (Joshi et al. 2001). Although the level of decrease varied among the tissues, the results in this study are consistent with the findings that X-ray- or γ-ray-irradiated mice showed a decreased folate level and an increase in degraded compounds (Endoh et al. 2006, Kesavan et al. 2003). It is therefore suggested that folate requirement is enhanced when oxidative stress is accumulated.

Folate decreased significantly in the bone marrow, but not in the liver. Previously, we reported that the degree of the decrease in vitamin C and vitamin E, and the increase in 8-hydroxydeoxyguanosine and 4-hydroxy-2-nonenal were lower in the liver than in bone marrow (Umegaki & Ichikawa 1994, Umegaki et al. 1995, 2001). We speculate that the increase of iron in the bone marrow after TBI, and high antioxidant system in the liver would underlie the

mechanisms (Umegaki et al. 2001). It has been shown that folate is degraded in the presence of iron and scavenges free radicals efficiently (Joshi et al. 2001, Shaw et al. 1989). It has also been shown that the decrease in the percentage of conjugated folate in bone marrow (from 42–10%) was lower than that in the liver (from 56–60%) in rats irradiated with X-ray (Viswanathan & Noronha 1970). Mono- and di-glutamic forms of folate are less likely to bind to folate-dependent enzymes and are catabolized (Suh et al. 2000). The form of folate between bone marrow and liver may be different, resulting in different catabolism due to TBI in this study. It has been shown that the microbiological assay used for folate measurement in this study can detect mono-, di-, tri-glutamic forms of folate (Tamura 1990), and that the cleavage of C^9-N^{10} bond of folate molecules by hydroxyl radical is a mechanism for the catabolism (Patro et al. 2005). However, it is unclear the types of damage to the folate molecules in various tissues due to TBI. Further study will be needed to clarify the underlying mechanisms.

TBI of a few gray is performed several times preceding bone marrow transplantation to kill the bone marrow cells of the recipient. Decreases in antioxidants such as vitamin E and beta-carotene in plasma were reported by TBI (Clemens et al. 1990), but little is known about folate. The results of this study suggest that folate in plasma is also decreased by TBI in humans. It is noted that folate levels affect the effectiveness and toxicity of cancer chemotherapy in animal experiments and *in vitro* studies (Whiteside et al. 2004). Sometimes, both radiotherapy and chemotherapy are performed simultaneously (Clemens et al. 1990), and the decrease in folate by radiotherapy may affect the therapeutic effects. Oxidative stress is induced in our body not only by irradiation, but also by chemical treatments or in pathological states such as diabetes mellitus. A decline in folate status increases the level of homocysteine (Ueland et al. 1993), which induces oxidative damage to the cells (Oikawa et al. 2003). Accordingly, it is suggested that folate status is involved in various diseases in many ways. It may be necessary to consider folate status during conditions involving oxidative stress, particularly during radiotherapy and to test whether excessive normal tissue morbidity following radiotherapy is related to folate status.

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日本骨粗鬆症学会 平成17年度 研究奨励賞

血中25-ヒドロキシビタミンDの新規定量法の開発と臨床応用

津川尚子　鎌尾まや　須原義智　岡野登志夫

はじめに

血中25-hydroxyvitamin D（25-OH-D）濃度はビタミンD栄養状態を最もよく反映する代謝物として骨代謝解析において欠かせない指標である。25-OH-D濃度は，従来HPLC法や競合的蛋白結合（CPBA）法で測定されてきたが，最近では特異的抗体を用いたradioimmunoassay（RIA）法や化学発光を用いた蛋白結合測定法（CLPBA）が用いられるようになった。しかし，これらの測定法はおのおのに原理が異なり，検体によっては異なる数値を与える場合がある。このことから，簡便な繁用法とは別に，標準法として真度，精度，感度がともに高い定量法の開発が望まれている。そこで，質量分析法を利用したLC/MS/MS法による25-OH-D_3，25-OH-D_2および24,25$(OH)_2D_3$濃度の高感度分別定量法を開発し，従来法による測定の妥当性を評価した。また，この方法を臨床検体に応用し，ビタミンD栄養の評価を行った。

1　方　　法

1）LC/MS/MS法による血中25-OH-D濃度測定法の確立

①血液サンプルからの抽出

ヒト血漿あるいは血清0.1mLを遠沈管にとり，

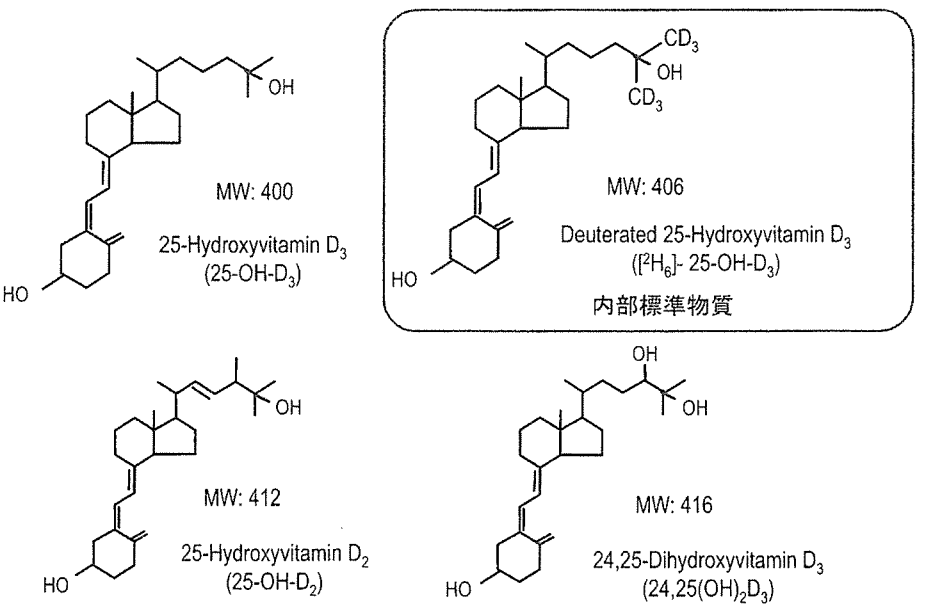

図1　ビタミンD代謝物および内部標準物質の化学構造

Key words：25-hydroxyvitamin D_3，25-hydroxyvitamin D_2，LC/MS/MS

神戸薬科大学衛生化学研究室

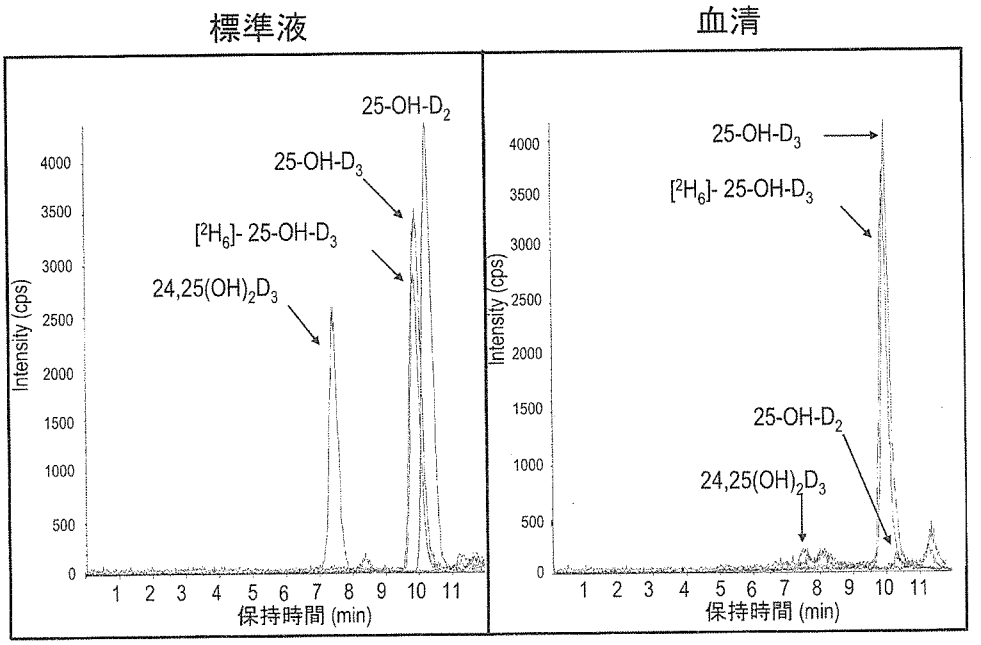

図2　LC/MS/MS分析におけるクロマトグラム

合成内部標準物質[1]として[$^2H_6$]-25-OH-D_3（側鎖26,27位メチルの水素を重水素化した化合物；図1）を2ng添加し，メタノール0.2mLを加えてボルテックスミキサーで撹拌した。3000rpmで遠心分離した後，得られた上清を，あらかじめメタノール/水（7：3，v/v）15mLで洗浄したBond Elut C_{18}に負荷し，メタノール/水（7：3，v/v）15mLで洗浄後，25-OH-D_2/D_3および24,25$(OH)_2D_3$画分をアセトニトリル/メタノール（8：2，v/v）5.0mLにより溶出させた。溶出液をロータリーエバポレーターで乾固した後，得られた残渣をメタノール100μLに溶解し，50μLを以下の条件のLC-APCI/MS/MSに適用した。別に調製したビタミンD代謝物標準溶液の分析を同時に行い，内部標準物質に対する標準ビタミンD代謝物のピーク面積比（Qs）を算出し，検量線を作成した。検体の分析から同様におのおののピーク面積比（Qt）を算出し，検量線よりLC-APCI分析時の濃度Aを求め，以下の計算により血中濃度を算出した。

血中濃度（ng/mL）＝A×20/50

②ビタミンD代謝物標準液の調製

25-OH-D_3，25-OH-D_2，24,25$(OH)_2D_3$（図1）を100ng/mL含む標準原液を調製し，この溶液から10～200ng/mLの範囲で段階的な希釈溶液を調製した。一方，重水素ラベルした[$^2H_6$]-25-OH-D_3を100ng/mL含む内部標準液を別に調製した。これと先の標準希釈液を等容量で混合し，25-OH-D_3，25-OH-D_2，24,25$(OH)_2D_3$を5～100ng/mLおよび内部標準物質50ng/mLを含む標準系列を得た。

③HPLC条件

ポンプ：LC-10AD（島津製作所社製），オートインジェクター：SIL-10AD（島津製作所社製），カラム：CAPCEL PAK C_{18} UG120（4.6×250mm，5μm，資生堂社製），移動相：メタノール：水（95：5，v/v），流速：0.5mL/min

④APCI-MS/MS装置およびMS検出条件

装置：API-3000（アプライドバイオシステムズ社製），MS検出条件：Precursor ion/product ion（*m/z*）：25-OH-D_3（*m/z*：401.4/257.0），25-OH-D_2（*m/z*：413.4/355.4），24,25$(OH)_2D_3$（*m/z*：417.4/363.1），[$^2H_6$]-25-OH-D_3（*m/z*：407.4/263.4）

⑤定量精度の確認

市販のヒトコントロール血清（和光純薬社製）を用いて，IntraおよびInter assayを行い，定量

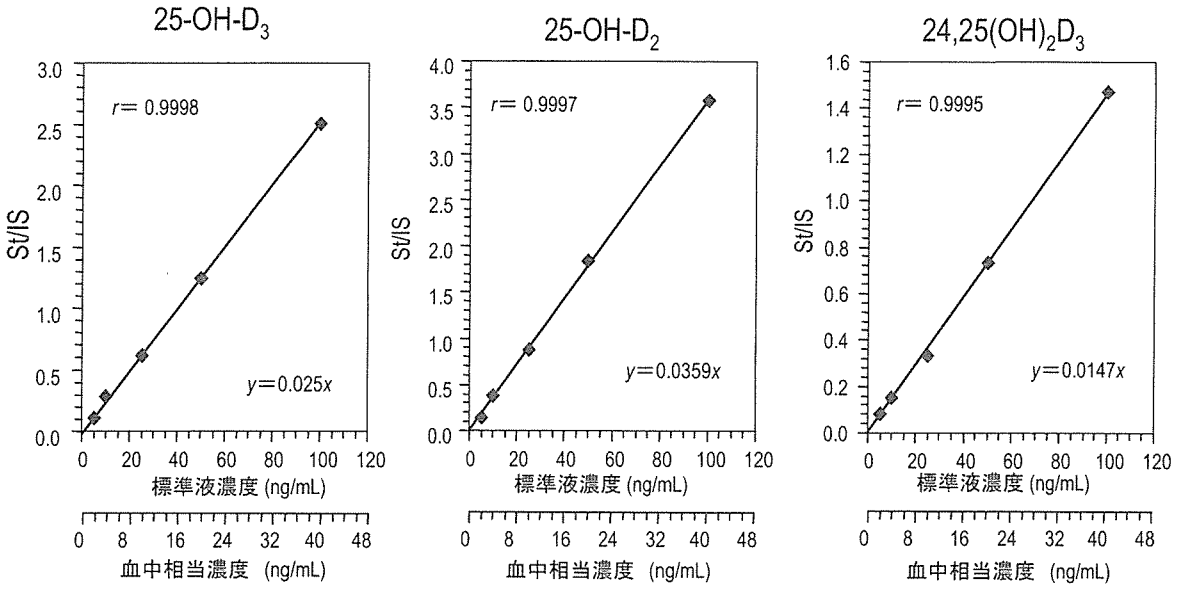

図3　ビタミンD代謝物の標準検量線

表1　標準血清を用いたビタミンD代謝物濃度の精度評価

代謝物		濃度	RSD%
25-OH-D_3	Intra assay（n=10）	20.0±1.1（ng/mL）	5.7
	Inter assay（n=5）	18.6±0.5（ng/mL）	2.5
	Recovery（n=5）	103.8±4.3（%）	4.1
25-OH-D_2	Intra assay（n=10）	2.5±0.1（ng/mL）	4.5
	Inter assay（n=5）	2.7±0.1（ng/mL）	5.1
	Recovery（n=5）	99.3±2.2（%）	2.2
24,25$(OH)_2D_3$	Intra assay（n=10）	2.8±0.3（ng/mL）	11.4
	Inter assay（n=5）	2.5±0.3（ng/mL）	9.9
	Recovery（n=5）	98.8±5.1（%）	5.2

平均値±標準偏差

精度を確認した。

2）RIA法との比較

長野県在住の高齢者を中心とする日本人女性278名（62.1±11歳）の血漿中ビタミンD代謝物濃度を，繁用法である市販の25-OH-D RIAキット（DiaSorin社製）を用いた測定値と比較した。使用したヒト血漿検体は成人病診療研究所の白木正孝先生よりご供与いただいた。

3）統計解析

MedCalc9.0.1.1を用いて解析した。

2　結　　果

図2に示すように，標準液ならびに標準血清において25-OH-D_2，25-OH-D_3，24,25$(OH)_2D_3$および内部標準物質は単一ピークとして検出された。標準溶液に対して作成した検量線は，5～100ng/mLの範囲で良好な直線性を示した（**図3**）。また，検出限界はいずれも血漿中濃度として1ng/mLであり十分な感度が得られた。

市販のヒトコントロール血清（和光純薬社製）を用いて，Intra assayおよびInter assayを行ったところ，**表1**に示すように十分な精度が得られ

表2　LS/MS/MS 法および RIA 法で測定されたヒト血漿中ビタミン D 代謝物濃度

LC/MS/MS				RIA (ng/mL)
25-OH-D_3 (ng/mL)	25-OH-D_2 (ng/mL)	24,25$(OH)_2D_3$ (ng/mL)	Total (ng/mL)	
21.2±6.5	0.8±1.4	1.1±0.6	23.1±6.9	20.4±6.5

n=278

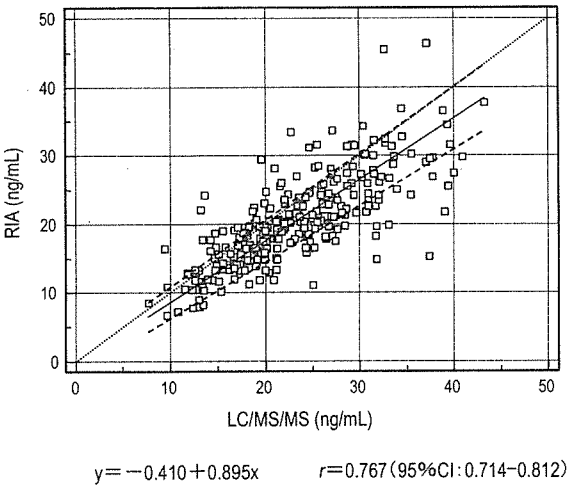

図4　Passing & Bablock 回帰分析

た。また，各ビタミン D 代謝物を 20ng/mL 添加して添加回収率を求めたところ，良好な回収率が得られた（**表1**）。血清の 3 段階希釈試験で得られた回帰直線の相関係数は 25-OH-D_3：0.9999，25-OH-D_2：0.9967，24,25$(OH)_2D_3$：0.9997 であった。以上のことから，本法における定量は十分な精度・真度であると判断した。

本法と従来法の測定値の比較を行うため，健常ヒト血漿278検体を用いて DiaSorin 社製 25-OH-D RIA キットによる測定値との比較を行った。それぞれの測定法から得られた平均±SD を**表2**に示す。LC/MS/MS 法で分別定量された 3 種の D 代謝物濃度の平均値を比較したところ，血中 25-OH-D_2 および 24,25$(OH)_2D_3$ 濃度は 25-OH-D_3 濃度の約 3〜5%程度で存在していることを確認した。RIA 法による 25-OH-D 濃度は 25-OH-D と 24,25$(OH)_2$D の合計値として得られるため，両測定法の比較では LC/MS/MS 法の濃度として 25-OH-D_3，25-OH-D_2，24,25$(OH)_2D_3$ の合計値を用いた。Passing & Bablock 回帰分析で両測定法の関係を評価した結果（**図4**），相関係数 r=0.767（95%CI：0.714〜0.812），直線回帰式 y(RIA)＝−0.410＋0.895x（LC/MS/MS）が得られ，LC/MS/MS 法による測定値はやや高値を示すものの両測定法には良好な相関関係が得られた。次に，ビタミン D の不足・欠乏の指標であり 25-OH-D 濃度とは逆相関することが知られる血中 PTH 濃度との関係を比較した結果，両者はほぼ同等の逆相関関係を示し，両測定法によるビタミン D 不足の評価に差異はないと判断された（**図5**）。また，両測定法による 25-OH-D 濃度は骨吸収マーカーの NTX とも有意に負相関することを確認した（LC/MS/MS：p=0.007，RIA：p=0.005）。

次に，LC/MS/MS 法で測定された 25-OH-D_3 濃度と 24,25$(OH)_2D_3$ 濃度の関係について検討した。24,25$(OH)_2D_3$ は 25-OH-D_3 の異化代謝物であり，両者が正相関することが知られているが，今回の対象者においても LC/MS/MS 法で測定された両代謝物濃度は有意な正相関関係を示すことを確認した（**図6**）。25-OH-D 濃度と PTH 濃度に有意な負相関があることから，PTH 濃度と 24,25$(OH)_2D_3$ 濃度間にも有意な負の相関関係を認めた（p=0.003，r=0.179）。一方，PTH は腎臓の 1α-水酸化酵素の誘導と 24-水酸化酵素の抑制作用を示すことが知られることから，24,25$(OH)_2D_3$/25-OH-D_3 比と血中 PTH 濃度の関係を解析した結果，両者は弱いながら有意な負の相関関係を示すことを確認した（p=0.011）。

3　考　　察

血中 25-OH-D の濃度は，従来 HPLC 法あるいは CPBA 法で測定されていた。しかし，これらの測定法は 0.5mL 程度の検体が必要で抽出・精製も比較的煩雑なため，熟練した技術と測定に長

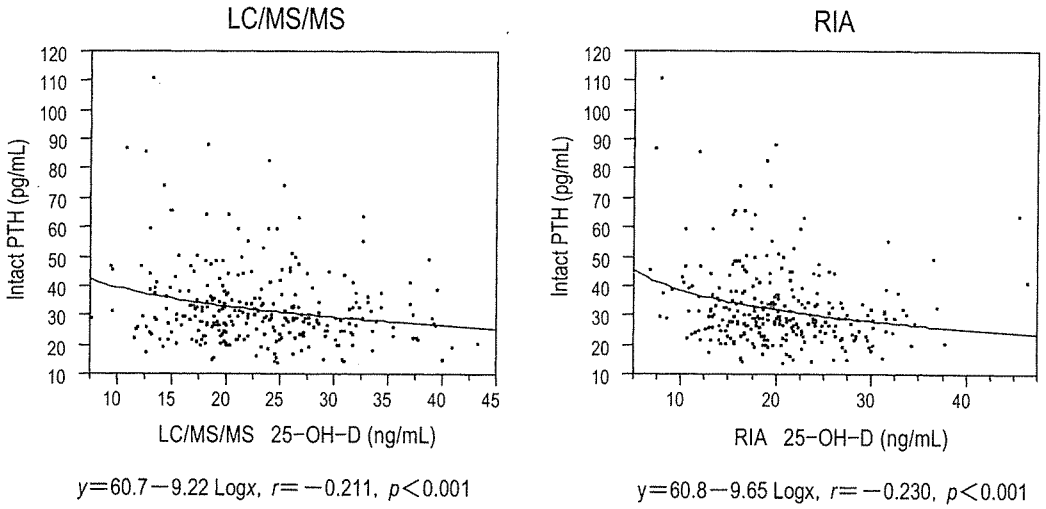

図5　LC/MS/MS 法および RIA 法による血中 25-OH-D 濃度と PTH 濃度との関係

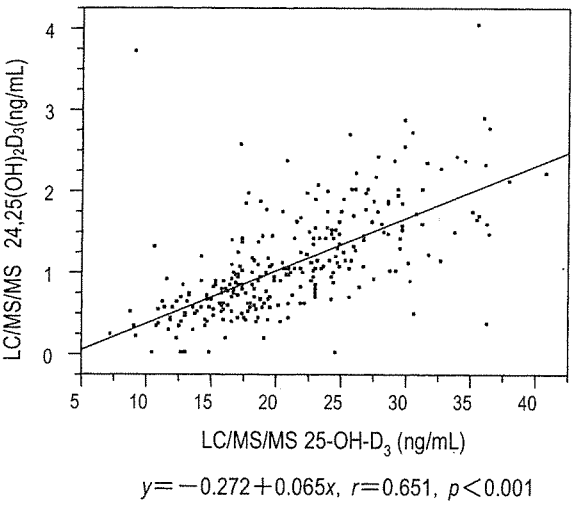

図6　血中 25-OH-D_3 濃度と 24,25$(OH)_2D_3$ 濃度の相関

時間を要することが難点であった。そのため，最近では RIA 法や酵素免疫（EIA）法や，CPBA 法と化学発光を利用した chemiluminescence protein-binding assay（CLPBA）法[2]，EIA 法と化学発光を利用した自動測定法[3]が利用されている。これらの方法はいずれも抗体やビタミン D 結合蛋白質（DBP）を利用した生物化学的方法であるが，検体中に含まれる種々の要因によってしばしば測定値に変動が生じることがある。標準法として物理化学的検出法である GC-MS 法[4]が用いられているが，今回新たに高精度質量分析法を用いた LC/MS/MS 法を開発し，本法が感度，精密度，正確度ともに十分な精度を備えた方法であることを確認した。このことから，本法は今後 HPLC 法や GC-MS 法に代わる標準法として利用できることが期待できる。RIA 法との比較においては，両者は良好な相関性を示し，両測定方法の妥当性を評価することができた。また，本法はビタミン D の栄養状態を十分評価できる方法であることも確認した。

25-OH-D_3，25-OH-D_2，24,25$(OH)_2D_3$ 濃度の同時分別定量は繁用法には期待できない LC/MS/MS 法のメリットである。抗体や DBP を用いる生物化学的検出法を用いる測定法の場合，25-OH-D_2 と 25-OH-D_3 に対する反応性の違いが問題となり，D_2 サプリメント服用者などにみられる 25-OH-D_2 濃度が高い血漿では測定法によって過大あるいは過少評価される場合がある[5~8]。わが国では米国のように D_2 をサプリメントとして利用するということはほとんどないが，シイタケなど D_2 含有食品の摂取が比較的多いために血中での存在量を把握しておく必要がある。今回の対象者の 25-OH-D_2 濃度の平均値は 25-OH-D_3 濃度の約 3%程度で測定値にはほとんど影響しない濃度であるものの，その範囲は 0～15.1ng/mL であったことから，一部の検体において分別定量による評価が必要となる可能性が示唆され

る。また，24,25$(OH)_2D_3$濃度については，副甲状腺機能や腎機能との関連も報告されており[9,10]，これらのD代謝物濃度の簡易分別定量が，D栄養とカルシウム代謝，骨代謝との関連性の臨床評価に応用できるものと期待される。

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Development of Novel Serum 25-Hydroxyvitamin D Determination Method and Its Clinical Application

Naoko Tsugawa et al.

Department of Hygienic Chemistry, Kobe Pharmaceutical University

25-hydroxyvitamin D (25-OH-D) can be used for the evaluation of vitamin D nutritional status. However, many reports have also pointed out the marked inter-laboratory variability in the serum 25-OH-D measurement, making it difficult to define the optimal serum 25-OH-D concentration for the maintenance of bone health. Therefore, we have developed a precise and reliable method to determine 25-hydroxyvitamin D (25-OH-D_2/-D_3) in human plasma using HPLC-tandem mass-mass spectrometry with atomospheric-pressure chemical ionization (LC/MS/MS).

Osteoporosis Japan

日本人高齢女性における血中ビタミンK濃度と骨折との関係

津川尚子[1)] 須原義智[1)] 鎌尾まや[1)]
岡野登志夫[1)] 田中 清[2)] 白木正孝[3)]

はじめに

ビタミンK(VK)は多くの血液凝固因子の合成において重要な役割をもつことが知られる。一方VKは，オステオカルシンやマトリックスグラ蛋白あるいはプロテインSのようなVK依存性蛋白質を，γ-カルボキシル化することによって骨代謝において重要な役割を果たす[1,2]。低フェロキノン(PK)摂取は，閉経後女性の大腿骨頸部骨折のリスクを増大させるとともに，大腿骨，脊椎における低BMDと関連があると報告されている[3〜6]。わが国では骨粗鬆症治療にVK_2が使用されているが，PKやメナキノン(MK-4, MK-7)を中心とするVK栄養が骨粗鬆症予防に果たす役割については十分な検討がなされていない。そこで，骨粗鬆症予防におけるVKの栄養効果を検討するため，日本人成人女性を対象に血中PKおよびMK-4，MK-7濃度を測定し，骨代謝関連指標との関連について調査した。

1 方　法

対象者は30〜88まで歳の日本人女性398名(30〜49歳：52名，50〜69歳：216名，70歳以上：130名)，平均年齢62.5歳である。骨粗鬆症以外の骨代謝疾患をもつ女性および活性型VD，VK，VK拮抗薬，エストロゲン，ビスフォスフォネート，ステロイドなどの骨代謝関連薬を服用している対象者は除外した。また，BMIが16.5以下の対象者は除外した。

測定項目は血中PK，MK-4，MK-7濃度，PTH，ucOC，Ca，P，BAP濃度，Alp活性，尿中NTX/Cr，DPD/Cr，U-Ca/Cr，BMI，L_{2-4}BMDおよび椎体骨骨折頻度である。統計解析には，JMP 5.0.1Jを用いた。

2 結果と考察

日本人高齢者女性398人の血漿中PK，MK-4，およびMK-7濃度はそれぞれ1.57±1.22，0.10±0.22と6.47±9.65ng/mLであった。他の血漿および尿中生化学パラメータは正常範囲内にあり，加齢に伴って血清ucOC濃度と尿中NTX，DPD濃度は増加し，L_{2-4}BMDは減少した。対象者のうち脊椎骨折有病者は72名(18.1%)であった。

血中PKとMK-7濃度は年齢層30〜49歳および70歳以上に比べて50〜69歳で有意に高く，血中ucOC濃度と逆相関した。**図1**に，VK濃度と脊椎骨折との関係を評価するロジスティク回帰分析の結果を示す。PK濃度の増加と骨折有病率の減少とに有意な関係を認めた。MK-7濃度の増加は有意ではないが骨折有病率を減少させる傾向にあったが，MK-4濃度は骨折有病率と

Relationship between Plasma Vitamin K Concentration and Bone Fracture in Japanese Elderly Women
Naoko Tsugawa : Department of Hygienic Sciences, Kobe Pharmaceutical University, *et al.*
Key words : Vitamin K, Bone fracture, Phylloquinone, Japanese women

[1)]神戸薬科大学衛生化学 [2)]京都女子大学家政学部食物栄養学 [3)]成人病診療研究所

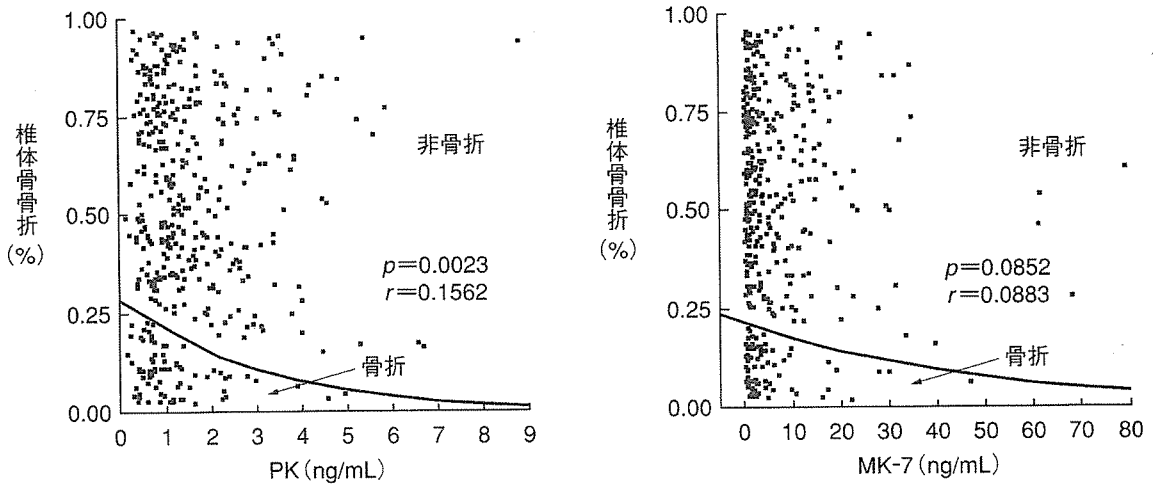
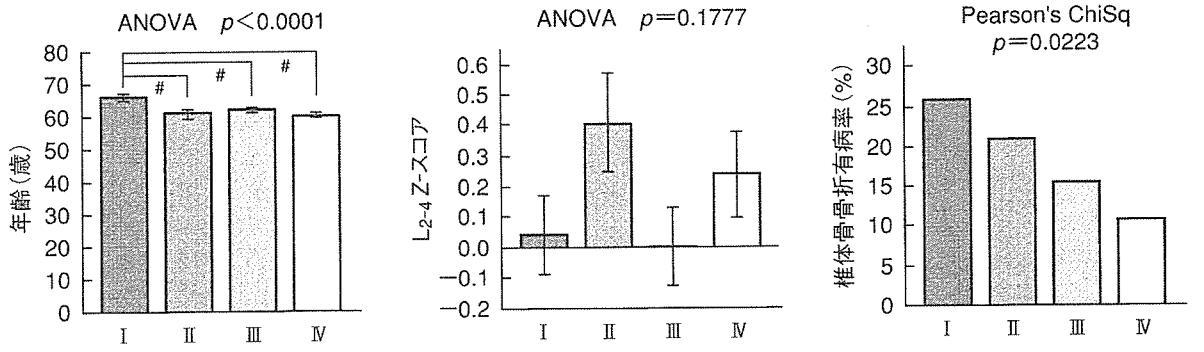


図1　ロジスティック回帰分析による血中ビタミンK濃度と椎体骨骨折の関係



群	オッズ比	95% 信頼区間	p値
Ⅰ：低 PK 高 ucOC	2.9	(1.4～6.2)	0.0041
Ⅱ：低 PK 低 ucOC	2.2	(1.0～5.0)	0.0505
Ⅲ：高 PK 高 ucOC	1.5	(0.7～3.6)	0.3096
Ⅳ：高 PK 低 ucOC	1.0		

図2　Ⅰ, Ⅱ, Ⅲ, Ⅳ群における年齢，骨密度，骨折有病率

関連しなかった。

次に，対象者をPK濃度とucOC濃度の中央値(PK：1.18ng/mL, ucOC：3.73ng/mL)で4群(Ⅰ群：低PK高ucOC群，Ⅱ群：低PK低ucOC群，Ⅲ群：高PK高ucOC群，Ⅳ群：高PK低ucOC群)に分割して骨折有病率を比較した(**図2**)。Ⅰ群の年齢は他の3群に比して有意に高く，Ⅱ, Ⅲ, Ⅳ群間の年齢に差異はみられなかった。低ucOCとして特徴づけられるⅡ群とⅣ群は高いL_{2-4} BMDおよびL_{2-4}BMD Z-scoreを示す傾向にあった。しかしながら，脊椎骨折有病率はⅠ群からⅣ群に向けて低下し，BMD，年齢とは無関係であった。また，Ⅳ群に対するⅠ群，Ⅱ群の脊椎骨折のオッズ比は有意に高く，VKの栄養状態が低下すると骨折の危険性が高くなることが示唆された。

さらに，各群における骨折と骨密度あるいは年齢の関係をロジスティック解析し，骨折有病率が25%となる年齢とL_{2-4}BMDを逆推定した。I群の平均年齢は4群のなかで最も高かったものの，ロジスティック解析の結果，逆推定された年齢はI群で最も若く(68.5歳，95%CI：60.9～73.7)，IV群で最も高かった(74.2歳，95%CI：67.8～92.0)。一方，逆推定されたL_{2-4} BMDはI群で最も高く(0.8964，95%CI：0.7851～1.0482)，IV群で最も低かった(0.7197，95%CI：0.2289～0.8640)。対象者を低PK濃度群(I & II群)と高PK濃度群(III & IV群)に分けると，逆推定年齢は低PK濃度群で68.2歳(95%CI：63.8～71.9)，高PK濃度群で72.4歳(95%CI：68.2～79.8)となり，これらの推定値は互いの95%信頼限界の範囲を超える値となった。また，逆推定L_{2-4} BMDは，低PK濃度群で0.8929(95%CI：0.8104～0.9828)，高PK濃度群で0.7707(95%CI：0.5969～0.8561)となり，これらの推定値もまた互いの95%信頼限界の範囲を超える値となった。

これらの結果より，低PK群では高PK群に比べて高骨密度，低年齢で骨折が起こりやすいことが示唆され，VKの栄養状態の低下により骨折の危険性が増加する可能性が示唆された。

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論文類別： Minireview

Determination of Fat-Soluble Vitamins in Human Plasma, Breast Milk and Food Samples – Application in Nutrition Survey for Establishment of "Dietary Reference Intakes for Japanese" –

Maya Kamao, Naoko Tsugawa, Yoshitomo Suhara and Toshio Okano*

Department of Hygienic Sciences, Kobe Pharmaceutical University, 4-19-1 Motoyamakita-machi, Higashinada-ku, Kobe 658-8558, Japan

* To whom correspondence should be addressed:
Department of Hygienic Sciences, Kobe Pharmaceutical University, 4-19-1,
Motoyamakita-machi, Higashinada-ku, Kobe, 658-8558, Japan
Tel: +81-78/441-7563, Fax: +81-78/441-7565
E-Mail: t-okano@kobepharma-u.ac.jp

SUMMARY

Dietary habits are an important risk factor for lifestyle-related diseases. To carry out a nutrition survey of fat-soluble vitamins, we developed determination methods of fat-soluble vitamins using liquid chromatography-atmospheric pressure chemical ionization/tandem mass spectrometry or high-performance liquid chromatography with fluorescence detection. In these methods, stable isotope-labeled compounds or vitamin K analogs with a saturated side-chain were used as internal standards. These methods have high sensitivity and sufficient accuracy, and we applied them in a nutrition survey about the status of fat-soluble vitamins in Japanese women. Plasma concentrations of 25-hydroxyvitamin D_3 [25(OH)D_3] and 25-hydroxyvitamin D_2 [25(OH)D_2] in healthy postmenopausal women (n=98) were 20.5 ± 7.9 and 0.4 ± 1.4 ng/mL, respectively. A significant negative correlation in plasma levels between 25(OH)D and parathyroid hormone was observed. For vitamin K homologs, plasma levels of phylloquinone (PK), menaquinone-4 (MK-4) and menaquinone-7 (MK-7) in Japanese women of various ages (n=1409) were 1.03 ± 0.90, 0.12 ± 0.28 and 6.71 ± 13.6 ng/mL, respectively. The mean total vitamin K intake of Japanese young women was about 230 μg/day, and 94 % of participants met the Adequate Intake of vitamin K for women aged 18-29 y in Japan, 60 μg/day. Moreover, we determined fat-soluble vitamins in breast milk collected from Japanese lactating women and revealed that the contents of all-*trans*-retinol, vitamin D_3, 25(OH)D_3, α-tocopherol, PK and MK-4 in breast milk were 0.39 ± 0.14 μg/mL, 0.10 ± 0.15 ng/mL, 0.08 ± 0.04 ng/mL, 3.96 ± 1.84 μg/mL, 3.56 ± 2.19 and 1.77 ± 0.68 ng/mL, respectively.

Key words—fat-soluble vitamins, vitamin D, vitamin K, nutrition survey

INTRODUCTION

In Japan, lifestyle-related diseases have been increasing with the advent of the aging society and it is acknowledged that dietary habits are an important risk factor for these diseases. Thus, a nutrition survey aimed at humans is needed as well as a study of the bioavailability, physiological function and metabolism of nutrients to obtain scientific information for the primary prevention of lifestyle-related diseases through the improvement of dietary habits and nutrition. We especially focused on vitamins D and K which are important fat-soluble vitamins for the prevention of osteoporosis.

It is well recognized that plasma or serum levels of 25-hydroxyvitamin D [25(OH)D] reflect the nutritional status of vitamin D in humans. Vitamin D is metabolized to 25(OH)D in the liver and subsequently to the active form of vitamin D, 1α,25-dihydroxyvitamin D [1α,25$(OH)_2$D], or the inactive form of vitamin D, 24,25-dihydroxyvitamin D [24,25$(OH)_2$D], in the kidney. In addition, it was demonstrated that vitamin D and its metabolites are also metabolized to their respective C-3 epimers [1-5]. Vitamin D_3, which is the form of vitamin D synthesized by vertebrates including humans, and vitamin D_2, which is the major naturally occurring form in plants, are both metabolized in a similar fashion. 25(OH)D binds to vitamin D-binding protein (DBP) in the blood and is the most abundant circulating metabolite of vitamin D with a concentration of 20-50 ng/mL under normal conditions [6]. Thus, the plasma or serum concentration of 25(OH)D is considered to be a good indicator of the cumulative effects of exposure to sunlight and dietary intake of vitamin D. Plasma or serum 25(OH)D concentration can be measured by high-performance liquid chromatography (HPLC) with an ultraviolet (UV) detector [7], competitive protein-binding assay (CPBA) [8], radioimmunoassay (RIA) [9] and enzyme immunoassay (EIA) [10]. In recent years, RIA and EIA have been widely used in many laboratories and hospitals because of their superior simplicity, rapidity and accuracy; however, these methods require high-quality control to ensure reliable results [11-15]. Moreover, conventional RIA measures 25(OH)D along with 24,25$(OH)_2$D because their antibodies exhibit 100 % cross-reaction with 24,25$(OH)_2$D.

Meanwhile, one of the most common nutritional indicators of vitamin K is the plasma concentration of phylloquinone (PK, vitamin K_1). PK is produced by plants and algae, and the other vitamin K form, menaquinones (MKs, vitamin K_2), is synthesized by bacteria. MKs

comprise a family of molecules distinguished from PK by unsaturated side-chains of isoprenoid units varying in length from 1 to 14 repeats [15]. Vitamin K is a cofactor for an enzyme that converts specific glutamyl residues in several proteins such as plasma clotting factors II (prothrombin), osteocalcin (bone Gla protein) and matrix Gla protein to γ-calboxyglutamyl (Gla) residues. These vitamin K-dependent proteins play crucial roles in blood coagulation and calcification. Several reports indicate an important role for vitamin K in bone health. The administration of vitamin K results in increased bone-mineral density (BMD) and reduced bone resorption in humans [16, 17]. In epidemiological studies, low dietary vitamin K intake was associated with an increased incidence of hip fracture [18, 19]; however, no large-scale nutrition survey of vitamin K has been conducted due to the low plasma concentration of vitamin K. There is still the problem with the accuracy of HPLC with fluorescence detection, which is usually used for the quantitation of plasma vitamin K.

Based on this background, we developed precise assay methods for vitamins D and K using liquid chromatography-atmospheric pressure chemical ionization/tandem mass spectrometry (LC-APCI/MS/MS) and HPLC with a fluorescence detector. Then, we applied these methods in a nutrition survey of Japanese women.

Development of Determination Method for Vitamin D

We established a precise and sensitive assay method to determine $25(OH)D_3$, $25(OH)D_2$ and $24,25(OH)_2D_3$ in human plasma using LC-APCI/MS/MS to provide a gold standard [20]. The method involves the use of deuterated $25(OH)D_3$ as an internal standard, which was synthesized in our laboratory. After the addition of the internal standard to 0.1 mL of plasma samples, methanol was added for protein removal. Vitamin D compounds were purified by C_{18} silicagel mini-column and detected by the MS/MS multiple reaction monitoring (MRM) method. The average spiked recoveries from authentic compounds added to normal human plasma samples for $25(OH)D_3$, $25(OH)D_2$ and $24,25(OH)_2D_3$ were 98-104 %. The average intraassay variation values (relative standard deviation) for $25(OH)D_3$, $25(OH)D_2$ and $24,25(OH)_2D_3$ were 5.7, 4.5 and 11.4 %, respectively. The average interassay variation values for $25(OH)D_3$, $25(OH)D_2$ and $24,25(OH)_2D_3$ were 2.5, 5.1 and 9.9 %, respectively. Mean plasma concentrations of $25(OH)D_3$, $25(OH)D_2$ and $24,25(OH)_2D_3$ in healthy postmenopausal

women (n=98) were 20.5 ± 7.9 (mean ± S.D.), 0.4 ± 1.4 and 0.5 ± 0.7 ng/mL, respectively. The concentrations of 25(OH)D measured by the RIA method using a DiaSorin RIA kit were well correlated with the concentrations of 25(OH)D plus $24,25(OH)_2D_3$ measured by the proposed method, although the RIA method gave slightly higher concentrations than the LC-APCI/MS/MS method. In addition, a significant negative correlation was observed between plasma levels of 25(OH)D and parathyroid hormone (PTH) with the LC-APCI/MS/MS method. In contrast, no significant correlation was observed in plasma levels between 25(OH)D and PTH with the RIA method. Plasma PTH level is an important indicator of vitamin D deficiency or insufficiency. Recently, a negative correlation between plasma 25(OH)D and PTH levels was reported from some cohort studies of healthy subjects [21, 22]. These results suggest that this LC-APCI/MS/MS method would be useful for the evaluation of vitamin D status and provide useful information in the diagnosis of vitamin D insufficiency/deficiency, as well as for the treatment and prevention of osteoporosis with vitamin D.

Development of Determination Method for Vitamin K

We also developed a determination method for vitamin K homologs including PK, MK-4 and MK-7 in human plasma using LC-APCI/MS/MS [23]. As internal standard compounds, ^{18}O-labeled PK, MK-4 and MK-7 were used. After the addition of internal standards to 0.5 mL of plasma samples, vitamin K compounds were extracted with ethanol and hexane. The average spiked recoveries from authentic compounds added to normal human plasma samples for PK, MK-4 and MK-7 were 98-102 %. The average intraassay and interassay variation values for PK, MK-4 and MK-7 were less than 10 %. The quantitation limits for PK, MK-4 and MK-7 were less than 3 pg per injection. Thus, we conclude that this novel LC-APCI/MS/MS method has enough reproducibility and sensitivity to measure vitamin K in human plasma; however, this method does not establish a universal routine assay as it uses an expensive measuring instrument. Therefore, we developed an improved HPLC fluorescence determination method for vitamin K homologs using post-column reduction and synthetic vitamin K analogs with different lengths of the saturated alkyl side-chain as internal standards [24]. Selectivity and reproducibility were increased by optimizing chromatographic conditions

including the mobile phase and excitation wavelength for MK-4 or less polar derivatives, PK and MK-7. The detection limits for PK, MK-4 and MK-7 were less than 4 pg per injection. The recoveries of PK, MK-4 and MK-7 were 93-105 % and the inter- and intraassay variation values of normal human plasma for PK, MK-4 and MK-7 were less than 10 %. The data showed good correlation between the proposed HPLC fluorescence determination method and the LC-APCI/MS/MS method for PK (r^2=0.979), MK-4 (r^2=0.988) and MK-7 (r^2=0.986) (Fig. 1). These results suggest that the improved HPLC fluorescence detection method allows the determination of vitamin K to evaluate the clinical and nutritional status as well as the LC-APCI/MS/MS method. Thus, this method was applied to plasma samples from Japanese women of various ages (n=1409). Plasma levels of PK, MK-4 and MK-7 were 1.03 ± 0.90, 0.12 ± 0.28 and 6.71 ± 13.6 ng/mL, respectively. The plasma levels of PK in elderly women (62.7 ± 10.9 y) were significantly higher than those of high school and junior high school girls. The plasma concentrations of MK-4 have a tendency to increase during periods of growth. In addition, plasma PK and MK-7 concentrations correlated inversely with undercarboxylated osteocalcin (ucOC) in elderly women [25]. The plasma PK or MK-7 concentration required to minimize the ucOC concentration was higher in the group over 70 y, and it decreased progressively for each of the younger age groups. Thus, circulating vitamin K concentrations in elderly people should be kept higher than those in young people.

Vitamin K Content of Foods and Dietary Vitamin K Intake in Japanese Young Women

In the current "Dietary Reference Intakes (DRIs) for Japanese", the Adequate Intake (AI) of vitamin K is set at 75 μg for adult men, 60 μg for women aged 18-29 y, and 65 μg for women 30 y and over as a probable sufficient quantity for the maintenance of normal blood clotting. However, the current AI might not be sufficient to maintain bone health. In addition, the assessment of dietary intake of both PK and MKs is incomplete in regions where people habitually eat fermented food, such as Japan. To obtain a closer estimate of dietary intake of PK and MKs in Japanese young women, PK, MK-4 and MK-7 contents in food samples (58 food items) were determined using an improved HPLC method with fluorescence detection. Next, we assessed dietary vitamin K intake in Japanese young women aged 20-23 y (n=125), using the vitamin K contents measured here and the Standard Tables of Food Composition in

Japan [26]. PK was widely distributed in green vegetables and algae, and high amounts were found in spinach and broccoli (raw, 498 and 307 μg/100g wet weight, respectively, unpublished data). Although MK-4 was widely distributed in animal products, overall MK-4 content was lower than PK. Relatively high amounts of MK-4 were found in chicken meat (raw, 27 μg/100 g) and the egg yolk of hen's eggs (raw, 64 μg/100 g). MK-7 was observed characteristically in fermented soybean products such as natto (939 μg/100 g). The mean total vitamin K intake of Japanese young women was about 230 μg/day and 94 % of participants met the AI of vitamin K for women aged 18-29 y in Japan, 60 μg/day. Mean daily intakes of PK, MK-4 and MK-7 (MK-4 equivalent value) were estimated as 155.9 ± 91.1, 16.9 ± 10.4 and 57.4 ± 83.7 μg/day, respectively. The contributions of PK, MK-4 and MK-7 (MK-4 equivalent value) to total vitamin K intake were 67.7, 7.3 and 24.9 %, respectively; therefore, PK from vegetables and algae, and MK-7 from pulses (including fermented soybean foods) were the major contributors to the total vitamin K intake of Japanese young women.

Nutrition Survey on Fat-Soluble Vitamins of Japanese Lactating Women

To estimate an infant's intake of fat-soluble vitamins, we determined their levels in breast milk collected from Japanese lactating women (n=51, age: 30.8 ± 4.4 y, post-partum day: 1.5 ± 1.2 m) by the LC-APCI/MS/MS method using stable isotope-labeled compounds as internal standards. It was reported that the concentrations of vitamin D and its metabolites in human breast milk were very low [27, 28]. Therefore, we used a derivatization method with a Cookson-Type reagent to improve ionization efficiency for the determination of vitamin D and its metabolites in LC-APCI/MS/MS analysis [29]. The contents of all-*trans*-retinol, vitamin D_3, 25(OH)D_3, α-tocopherol, PK, MK-4 and MK-7 in breast milk were 0.39 ± 0.14 μg/mL, 0.10 ± 0.15 ng/mL, 0.08 ± 0.04 ng/mL, 3.96 ± 1.84 μg/mL, 3.56 ± 2.19, 1.77 ± 0.68 ng/mL and 1.19 ± 1.54 ng/mL, respectively (Table 1). Daily intake of vitamin D calculated from an infant's consumption of breast milk, 780 mL/day was 0.47 μg, which did not meet current DRIs (AI, 2.5 μg/day). The concentrations of all-*trans*-retinol, β-carotene, 25(OH)D_3, α-tocopherol, PK and MK-4 in breast milk were positively correlated with lipid content; thus, the secretion of fat-soluble vitamins in breast milk is thought to be highly influenced by lipids.