Analysis of Folate by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry

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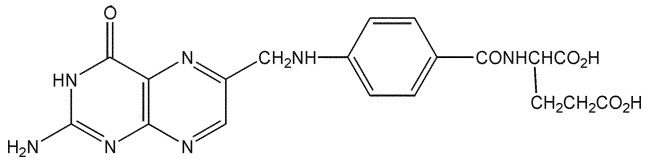
Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was used to observe folic acid and its derivatives such as tetrahydrofolate and 5-methyltetrahydrofolate in a vitamin tablet and in foods. Folic acid in a vitamin tablet was determined using angiotensin I as an internal reference. Tetrahydrofolic acid, 5-methyltetrahydrofolic acid, and an oxygenated folate were observed from a human blood sample using graphite plate. The results show that these mass spectrometric methods are useful for quickly obtaining a profile of folates.

Key Words : Folate, MALDI-TOF MS, Graphite plate, Vitamin, Blood

Introduction

Folate refers to a group of compounds based on the parent compound folic acid (FA; pteroylglutamic acid, structure I). FA is an essential vitamin involved in many important biochemical reactions. Recently, FA has received much attention due to its prophylactic role in the treatment of several diseases. The FA derivatives differ with respect to the state of oxidation of the pteridine ring, the number of glutamate residues conjugated to the *para*-amino-benzoic acid moiety, and the type of substitution at the 5- and/or 10-carbon position.[1,2]



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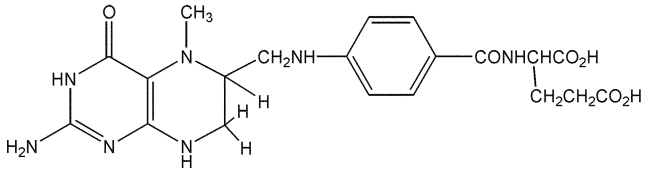

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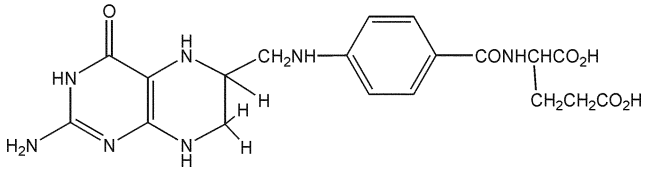

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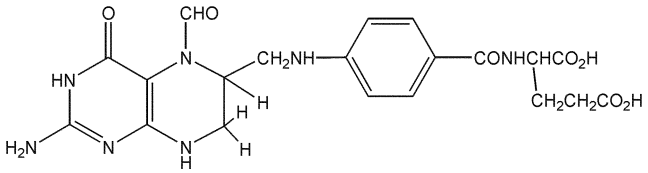

(IV)

Only reduced folates are found naturally in plants and animals. In humans, the predominant form of folate in the blood is 5-methyltetrahydrofolic acid (MTHF, II), whereas tetrahydrofolic acid (THF, III) is the major circulating form in the porcine species. Several different forms have been found in foodstuffs, the most common folates being MTHF, 5-and/or 10-formyltetrahydrofolic acid (folinic acid, CHOTHF, IV) and THF.[3] These dietary folates are converted to THF during absorption from the intestinal tract so that only THF is eventually transported to the tissues. THF forms the prosthetic group of many enzymes that are involved in the transfer of 'one carbon' moieties in amino acid and nucleotide metabolism, conversion of homocysteine to methionine, methylation of transfer RNA, and *de novo* purine nucleotide synthesis.[2] Prokaryotic cells can produce FA from other metabolites, while eukaryotes cannot and thus eukaryotes require FA in their diet.[4] Polyglutamates based on MTHF predominate in fresh food, but on storage these break down slowly to monoglutamates and are oxidized to less available folates.

The richest source of folates is liver, where most are present as the more available MTHF. Most other foods predominantly contain polyglutamates. Yeast extract, wheat bran and wheat germ, egg yolk, some cheeses and green leafy vegetables are rich sources of folates in addition to liver.[2,6] Bioavailability of the different forms and conjugates of FA are still poorly understood. Therefore, there is a need to separately determine the amount of different folates in foods and biological samples.

Folate is usually measured by cumbersome microbiological assay. Many estimates of the folate content of foods by microbiological assay are unreliable. The widely used folacin assay is a microbiological assay using *Lactobacillus casei* and it allows measurement of total folates at the nanogram level.[7] This method, however, is time-consuming and lacks reproducibility and specificity for various folate forms.[8]

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Several researchers introduced chromatographic separation prior to microbiological assay.[9] A number of chromatographic methods have been reported in which the folates are separated as their corresponding monoglutamates by conventional reversed-phase separation or by ion-pairing techniques with UV and/or fluorescence detection, and, in some instances, as intact polyglutamates.[10-14] Dual detection is required because some folates, *e.g.* FA, do not respond to fluorescence detection. Many of the folates are extremely sensitive to oxidation and require antioxidants such as ascorbic acid and/or 2-mercaptoethanol in the sample preparation. Moreover, these compounds can cause interference.[3]

Mass spectrometric methods offer attractive alternatives. Recently, a liquid chromatography-electrospray ionization (ESI) mass spectrometric method for analysis of FA, THF, and CHOTHF has been reported.[3] It offers superior specificity and lower detection limit than conventional LC detection methods. Arnold and Reilly reported analysis of tetrahydrofolylpolyglutamic acid in bacteria cells by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS).[15] However, their analysis did not include low molecular weight folates such as FA, THF, MTHF, or folinic acid.

Generally, the low m/z region of a MALDI mass spectrum is crowded by the matrix peaks. Recently, it was demonstrated in our laboratory that graphite plate laser desorption/ionization time-of-flight mass spectrometry (GPLDI-TOF MS) could be used to observe low molecular weight nonpolar compounds without matrix complications.[16] In this paper, we report analysis of low molecular weight folates in a vitamin tablet, foods, and blood by MALDI- and GPLDI-TOF MS.

Semiquantitative analysis of folate was also of interest. MALDI-TOF MS has limitations for quantitative analysis, because the reproducibility of signal intensity in MALDI-TOF MS is not sufficient probably due to the sample inhomogeneity on the plate and the resulting variability in desorption/ionization efficiency. However, several articles have been published for quantitative analysis using MALDI-TOF MS. Abell *et al.* used MALDI MS for quantitative analysis of α-chaconine and α-solanine using tomatine as an internal standard.[17] Sugiyama et al. applied MALDI MS to quantitative analysis of serum sulfatide using hydrogenated N-acetyl lysosulfatide as an internal standard.[18] Bruenner *et al.* described concentration-dependent desorption/ionization of a 21-base oligonucleotide (MW 6361) using a 36-base oligonucleotide (MW 11,131) as an internal standard.[19] Preliminary results from semiquantitative analysis of folate by MALDI-TOF MS are also reported.

Experimental Section

Materials. Folic acid, tetrahydrofolic acid, 5-methyltetrahydrofolic acid disodium salt, dihydrofolic acid and angiotensin I were obtained from Sigma (St Louis, MO, USA). 2-Mercaptoethanol was from Bio-Rad (Hercules, CA, USA). Ascorbic acid was from Ducksan Pharmaceutical Co. (Ansan, Kyongkido, Korea). α-Cyano-4-hydroxycinnamic acid (CHCA) was from Aldrich Chemical Co. (Milwaukee, WI, USA). All other chemicals were reagent grade. Distilled water was prepared using a Barnstead purification system (Dubuque, IW, USA). Spinach and vitamin tablet were purchased from a local market.

Standard Solutions. Stock standard solutions (1 mg/mL) were prepared by dissolving 50 mg folate in a mixture of 15 mL 0.1 M sodium hydroxide and 35 mL 0.1% TFA in water/acetonitrile mixture (2 : 1) containing 0.1% 2-mercaptoethanol. Nitrogen gas was bubbled through these standards for 15 seconds. The stock solutions were protected from light. Working standards were prepared by diluting the stock solution as necessary in the water/acetonitrile mixture used above.

Sample Preparation. A 0.1-2 g aliquot of a homogenized sample (spinach or vitamin tablet) was weighed into a 15 mL polypropylene tube. Ten mL of extraction buffer (0.1 M sodium acetate containing 1%(w/v) ascorbic acid and 0.1%(v/v) 2-mercaptoethanol, adjusted to pH 4 with glacial acetic acid) was added to each tube. Nitrogen gas was bubbled through the sample for 15 seconds. The folates were extracted by placing the tube in a boiling water bath for 1 h. After cooling, the samples were centrifuged at 10000 *g* for 20 minutes. The clear supernatant from each sample was filtered using a 0.22 μm nylon filter (Alltech, Cameo, USA).

Whole blood hemolysate samples were prepared in Dr. D. W. Lee's laboratory, Soon Chun Hyang University Hospital, Seoul, Korea as follows. Blood was collected in a tube containing 10 mM EDTA in deionized water. 0.1 mL of the blood sample was added to 2 mL of 1% (w/v) ascorbic acid solution. The mixture was gently inverted repeatedly to mix without foaming. For complete hemolysis, the mixture was then kept at 39.2 °C protected from light for 60 min. Hemolyzed specimens were stored frozen (−20 °C). Before preparing samples for GPLDI-MS analysis, the blood samples were filtered using a 0.22 μm nylon filter (Alltech, Cameo, USA).

Mass Spectrometry. Voyager Biospectrometry workstation with a linear mass analyzer (PE Biosystems, Framingham, MA, USA) was used, which was equipped with a 337 nm nitrogen laser and a 1.2 m flight tube. Mass spectra were obtained in the positive ion mode. 25 kV accelerating voltage was used. CHCA and angiotensin I were used for calibration. The graphite plate was custom-made, using commercial 7-μm graphite particles, identical in dimensions to the stainless steel plate provided by the manufacturer of the MALDI-TOF mass spectrometer used.

For quantitative analysis of folates, angiotensin I (1296.5 Da) was used as an internal standard. MALDI standards were prepared by mixing 1 μL working standard solution and 1 μL 0.77 M angiotensin I solution with 8 μL matrix (CHCA in 2 : 1 0.1% TFA/acetonitrile). A 1.5 μL aliquot of this mixture was applied to the stainless steel plate. Stainless steel plate was used because 2-mercaptoethanol in samples is adsorbed to the gold plate. MALDI samples of spinach

and vitamin tablet were prepared similarly using 1 μL extract instead of the working standard. GPLDI samples for whole blood hemolysate were prepared by mixing 5 μL whole blood hemolysate with 5 μL acetonitrile and applying 3 μL of this mixture to the graphite plate or by applying 3 μL of whole blood hemolysate directly to the graphite plate.

Results and Discussion

Observation of FA without Matrix. The structure of folate suggested that desorption/ionization could take place without a matrix. Figure 1 shows a mass spectrum obtained, using a stainless steel plate, from a saturated solution of authentic FA without matrix. $[M+H]^+$, $[M-Na]^+$, $[M-K]^+$ ions were observed at m/z 442.4 (expected, 442.4), 463.3 (expected, 464.4), and 479.5 (expected, 480.5), respectively. A mass error of about 0.2% was typical of the linear instrument used.[20] In the absence of the acidic matrix, the sodiated peak was more intense than the protonated peak. Even though FA could be observed without a matrix, the sensitivity was not sufficient for folate analysis in biological samples. Also angiotensin I, we used as an internal standard, needed a matrix for desorption/ionization. Therefore, CHCA matrix was used subsequently. Sensitivity of folate analysis was also enhanced significantly by the CHCA matrix.

Semiquantitative Analysis of Folate. Our primary interest was the possibility of using MALDI-TOF MS in qualitative analysis of different forms of folate in biological samples, since microbiological folate assay measures total folate and is not suitable for investigating the distribution of different folates in various samples.

The signal intensity for FA at varying concentrations was measured at a constant angiotensin I concentration of 0.10 ng/μL (77 nM) in the sample/matrix mixture being loaded on the stainless steel plate. Angiotensin I was used as an internal standard because it produces a very strong peak and it does not overlap with folate or its glutamates in the mass spectrum.

Figure 2 shows a mass spectrum obtained from a mixture

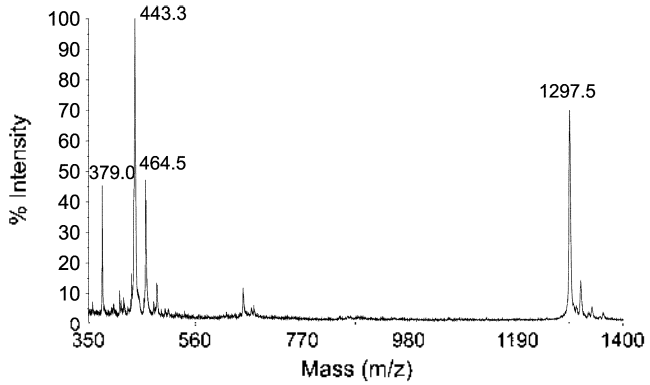


Figure 2. MALDI mass spectrum of folic acid (FA) with CHCA as matrix. The m/z peaks are CHCA dimer (379.0), $[FA+H]^+$ (443.3), $[FA+Na]^+$ (464.5), and [angiotensin I+H]$^+$ (1297.5).

containing 40 ng of FA and 0.10 ng of angiotensin I per μL using CHCA as matrix. The peak intensity ratio suggests that angiotensin I is much more efficiently desorbed/ionized and detected than FA. $[FA+H]^-$ and $[FA+Na]^-$ were observed at m/z 443.3 (expected at 442.4) and 464.5 (expected at 464.4), respectively. Again the mass error is about 0.2% typical of the instrument used.[20] The peak at m/z 486.6 might be due to a disodium adduct ion. The peak at m/z 379.0 is due to a CHCA dimer. Protonated angiotensin I is shown at m/z 1297.5 as expected. A similar mass spectrum was obtained from MTHF. From MTHF both $[MTHF+H]^+$ and $[MTHF-Na]^+$ were observed at m/z 459.8 and 481.7, respectively (result not shown).

The calibration curve in Figure 3 was obtained using sample mixtures containing 0.4, 1.0, 2.0, 4.0, 13, and 27 ng of FA, in addition to 0.10 ng of angiotensin I, per of the sample-matrix mixture loaded on the plate. The ratio of peak height for FA and angiotensin I gave a reasonably good linear fit indicating that the peak height ratio is approximately proportional to the concentration of FA and thus angiotensin I is a good internal standard for FA. The correlation coefficient was higher for $[FA+Na]^+$ ($R^2 = 0.997$) than for $[FA+H]^-$ ($R^2 = 0.968$) probably reflecting the sodium-rich environment.

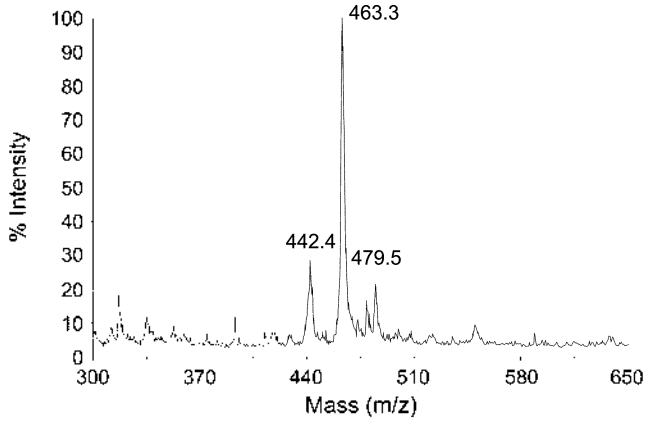


Figure 1. MALDI mass spectrum of folic acid (FA) without matrix. The m/z peaks are $[FA+H]^+$ (442.4), $[FA+Na]^-$ (463.3), and $[FA-K]^-$ (479.5).

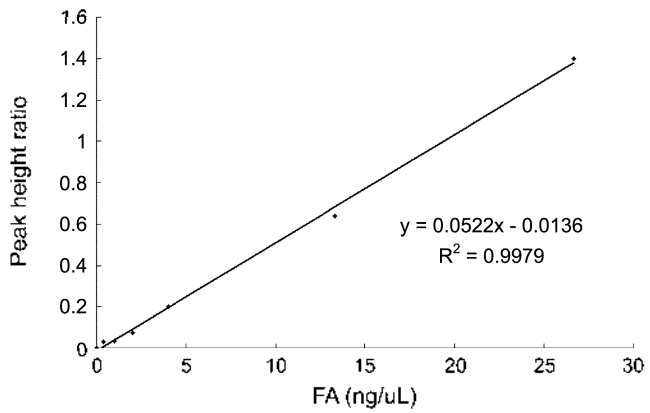


Figure 3. Calibration curve showing the peak height ratio (FA/angiotensin I) as a function of the amount of FA in the FA-matrix mixture loaded on the plate.

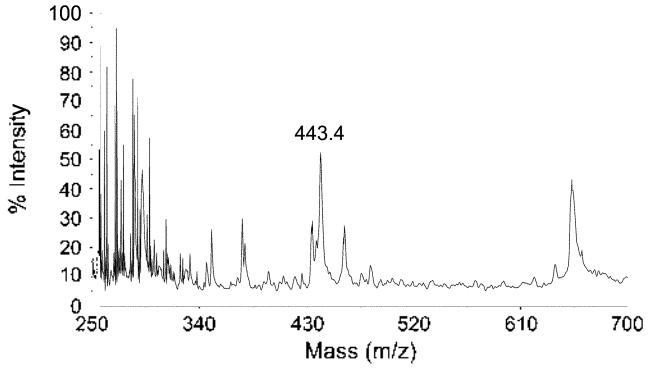


Figure 4. MALDI mass spectrum of a vitamin extract showing folic acid at 443.4.

Therefore, the sodiated peak was used in all quantitative work. The calibration was also better for $[MTHF+Na]^+$ (R^2 – 0.992) than for $[MTHF+H]^-$ (R^2 – 0.958).

Folate in a Vitamin Tablet. Figure 4 shows a MALDI mass spectrum obtained by dissolving a vitamin tablet in 100 mL water and diluting the solution 1000-fold before loading. $[FA+H]^-$ and $[FA+Na]^+$ peaks were observed at m/z 443.4 and 464.8, respectively. Even though several strong peaks were observed below m/z 300, no other peaks overlapped the FA peaks. Extraction and mass spectrometric analysis of folate were repeated five times, and based on internal calibration the average content of FA was calculated to be approximately 530 μg/tablet (18% standard deviation). This result is in reasonable agreement with the value on the label (400 μg/tablet) considering that the amount of folate added to the tablet would be higher (to avoid the possibility of non-compliance) than the amount indicated on the label. Peaks corresponding to THF or any other folate derivatives were not observed. Clearly, FA, not THF or MTHF, was used in the vitamin tablet. A vitamin tablet from another company yielded a similar result showing FA only. These results represent first observation of low molecular weight folate by MALDI MS and show that MALDI MS with internal calibration can be used for a rapid semiquantitative determination of folate in a fairly simple sample matrix such as a vitamin tablet.

Folate in Foods. Certain foods such as wheat bran or spinach are known to be rich in folate. However, only total folate level is usually reported. It is well known that only reduced folates are found in plants and animals. Figure 5 shows the MALDI mass spectrum obtained from spinach extract. As expected FA was not observed; however, THF and MTHF were observed at m/z 445.5 (expected 446.4) and 460.6 (expected 460.5), respectively. Folate with 1 glutamate was also observed at m/z 593.4 (V). In some cases folates with 4 or 5 glutamates were observed.

MALDI spectrum of spinach extract, however, had a high background and, therefore, quantitation of folate was difficult due to interference from overlapping peaks. Sample pretreatment and concentration might be necessary for quantitative folate determination from real food samples.

Both FA and THF, but not MTHF, were observed from

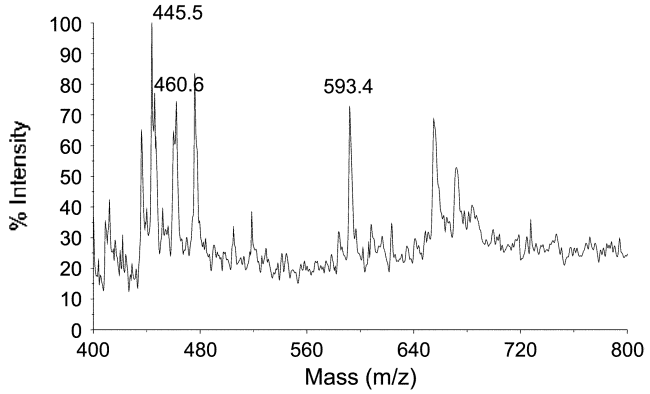
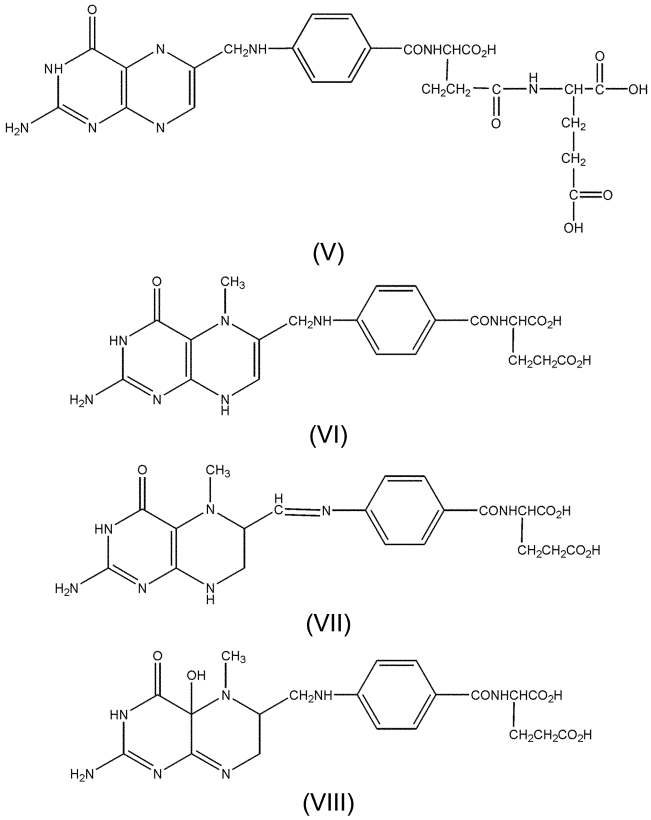


Figure 5. MALDI mass spectra obtained from spinach extract using CHCA as matrix. The peaks correspond to $[THF+H]^+$ (445.5), $[MTHF+H]^-$ (460.6), $[PteGlu_2-Na]^+$ (593.4).



corn flakes and from citrus juice. The results show that the method is useful for qualitative analysis of the folate species present in foods.

Folate in Blood Using Graphite Plate. The major folate of human blood is MTHF.[1] Microbiological assay still remains the most widely used method for determination of folates in human blood. Microbiological assay does not detect biological inactive modified folates such as oxidized folates.[1,15] In the case of MTHF, possible oxidized forms in human blood are biologically inactive 5-CH_3-5,6-H_2folic acid (VI), 9,10-dehydro-5-CH_3-5,6-H_4THF (VII), and 4a-OH-5-CH_3-4a,5,6,7-THF (VIII).[21]

Figure 6 shows GPLDI-TOF MS spectrum of the hemolyzed whole blood sample. The MALDI mass spectrum

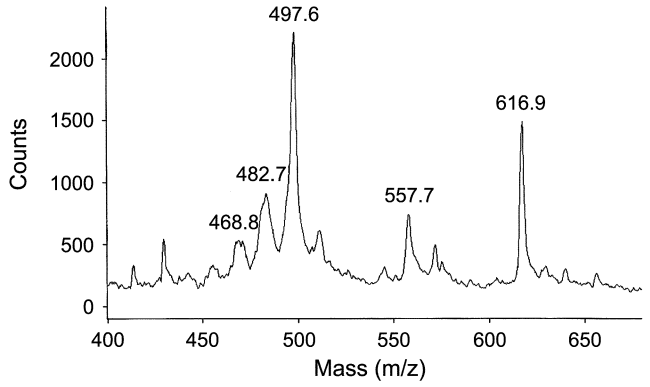


Figure 6. GPLDI mass spectrum of hemolyzed whole blood sample. The peaks correspond to $[THF+Na]^+$ (468.8), $[MTHF-Na]^+$ (482.7), $[(VIII)+Na]^+$ (497.6).

from the whole blood sample showed a higher sensitivity than the spectrum obtained by the graphite method (result not shown). However, the background peaks complicated the spectrum. This difference suggests that more chemical species in the blood are desorbed/ionized with the aid of the matrix, as in the MALDI method, than without the matrix, as in the GPLDI method. Obviously, there are plenty of polar compounds in the blood readily desorbed by the MALDI process and, therefore, use of the graphite method is advantageous for observing folate in blood.

In the past, graphite mixed with a liquid matrix, such as glycerol, was found to be useful in obtaining time-of-flight mass spectra of proteins,[22] 2500 Da polystyrene, and 3000 Da poly(ethylene glycol).[23] As a matter of fact, glycerol was used in the early mass spectrometric observation of proteins by Tanaka.[24] Direct use of graphite without a matrix was attempted by Zumbuhl *et al.*[25] Kim and Kang performed LDI-TOF MS of 2000 Da polypropyleneglycol and 2500 Da polystyrene using a 2-mm-thick graphite plate without a matrix.[26] Kim *et al.* obtained a detailed molecular weight distribution of poly(methylsilsesquioxane)s in the 100-1000 Da range by GPLDI-TOF MS.[16]

As shown in Figure 6, it appears that a significant portion of folates in the human blood exist as oxidized folates. The peaks at m/z 468.8 and 482.7 are believed to be $[THF+Na]^+$ and $[MTHF+Na]^+$, respectively. It is well known that sodium adducts are observed predominantly when graphite plate is used.[16] However, the strongest peak at 497.6 appears to be the sodium adduct of oxidized MTHF (VIII). The peaks at 557.7 and 616.9 were not identified. Thus MTHF and oxidized MTHF represent majority of folate in blood as expected.

It is not known whether the oxidized form could be reduced and utilized as dehydroascorbic acid is as vitamin C. If so, the microbiological assay might underestimate the folate content and it is desirable to include oxidized folate in the assay.

Conclusion

This study has demonstrated the applicability of MALDI-TOF MS for rapid folate analysis. Advantages of MALDI- and GPLDI-TOF MS include the speed of analysis and the capability to provide folate profile directly. MALDI-TOF MS method has a potential for semiquantitative folate analysis. Modification of the sample preparation to eliminate interference would be desirable for quantitative analysis. The establishment of a MALDI- and GPLDI method for the key folates will enable further detailed investigation into the problems associated with the physiological role of various folate species.

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