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Gas chromatographic–tandem mass spectrometric quantification of human plasma and urinary nitrate after its reduction to nitrite and derivatization to the pentafluorobenzyl derivative

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Abstract

Gas chromatography–mass spectrometry (GC–MS) of nitrite as its pentafluorobenzyl derivative in the negative-ion chemical ionization mode is a useful analytical tool to quantify accurately and sensitively nitrite and nitrate after its reduction to nitrite in various biological fluids. In the present study we demonstrate the utility of GC–tandem MS to quantify nitrate in human plasma and urine. Our present results verify human plasma and urine levels of nitrite and nitrate measured previously by GC–MS.

Keywords: Tandem mass spectrometry; Derivatization, GC; Nitrate; Nitrite

1. Introduction

Analysis of nitrite and nitrate by gas chromatography–mass spectrometry (GC–MS) requires conversion of these anions to volatile derivatives. In principle, this can be accomplished by two reactions, i.e., (a) sulfuric acid-catalyzed nitration of aromatics such as benzene and trimethoxybenzene to form nitrobenzene and nitrotrimethoxybenzene (NTMB) [1], and (b) by reaction of nitrite with pentafluorobenzyl (PFB) bromide to form a PFB derivative [2]. Analysis of nitrite as NTMB derivative and of nitrate as PFB derivative requires oxidation of nitrite to nitrate and reduction of nitrate to nitrite prior to derivatization, respectively. Negative-ion chemical ionization both of NTMB [3] and the PFB derivative [2] leads to strong fragmentation yielding nitrite (m/z 46) as the most abundant anion in both cases and less intense ions due to M^- and $[M-1]^-$, respectively. We have previously shown that analysis of nitrate as NTMB derivative in biological fluids is possible by GC–tandem MS by subjecting the parent ion M^- to collision-induced dissociation (CID) [3]. In the present work, we show the utility of GC–tandem MS to quantify nitrate in human urine and plasma as PFB derivative after reduction of nitrate to nitrite by cadmium.

2. Experimental

2.1. Materials and chemicals

Sodium [^{15}N]nitrate (98 atom% at ^{15}N) was

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obtained from Sigma (Munich, Germany). Sodium [^{15}N]nitrite (98 atom% at ^{15}N) was bought from Cambridge Isotope Laboratories (Andover, MA, USA). Sodium nitrite, sodium nitrate, toluene, ammonium chloride and cadmium powder (100 mesh) were purchased from Merck (Darmstadt, Germany). 1,2,3,4,5-Pentafluorobenzyl (PFB) bromide was obtained from Aldrich (Steinheim, Germany).

2.2. Reduction and derivatization procedures

Procedures for reduction of nitrate to nitrite by cadmium and derivatization of standards nitrite and nitrate and human plasma and urine samples was performed as described elsewhere [4] using [^{15}N]nitrate as internal standard at 40 μ*M* for plasma and 800 μ*M* for urine samples. Each three aliquots (100 μl) of buffer, i.e., 5 wt% ammonium chloride buffer, pH 8.8, were spiked with 40 and 800 μ*M* of [^{15}N]nitrate to determine the blank nitrate present in the reagent and solutions used. Accurately weighed cadmium powder (10 mg) was added to the samples (100 μl; aqueous solutions, plasma or urine samples, all diluted 1:10, v/v, with the ammonium chloride buffer), and reduction was performed by shaking for 90 min at room temperature. To the supernatants were added acetone (400 μl) and PFB bromide (10 μl), and the reaction mixtures were allowed to stand at 50°C for 60 min. Acetone was removed under nitrogen and reaction products were extracted by vortex-mixing with toluene (1 ml) for 1 min.

2.3. GC–MS and GC–tandem MS

GC–MS was performed on a Hewlett-Packard (HP) MS engine 5890A connected directly to a gas chromatograph 5890 series II equipped with an autosampler HP model 7673 (Waldbronn, Germany). GC–MS and GC–tandem MS were carried out on a Thermoquest (TSQ) 7000 apparatus (San Jose, CA, USA) connected directly to a TSQ Carlo Erba Instruments gas chromatograph Trace 2000 equipped with an autosampler model AS 2000. Two fused-silica capillary columns were used: An Optima 17 (15 m×0.25 mm I.D., 0.25 μm film thickness) from Macherey-Nagel (Düren, Germany) in the HP instrument and a DB-5MS (30 m×0.25 mm I.D., 0.25 μm film thickness from J and W Scientific (Rancho Cordova, CA, USA) in the TSQ instrument. The following temperature program was used in GC–MS and GC–tandem MS analyses: the column was held at 70°C for 1 min then increased to 280°C at a rate of 30 C°/min. Other GC–MS conditions were as described elsewhere in detail [2]. In GC–tandem MS analyses helium (at a constant pressure of 70 kPa) and methane (530 Pa) were used as carrier and reactant gases, respectively. For CID argon (0.130 Pa) was used at a collision energy of 25 eV. Electron energy and electron current were 230 eV and 300 μA, respectively. Injector, interface and ion-source were held constant at 200°C, 280°C and 180°C, respectively. Aliquots (1 μl) were injected in the splitless mode in both instruments. PFB derivatives of unlabeled and ^{15}N-labeled nitrate and nitrite had practically identical retention times on both columns (about 2.9 min in GC–MS and about 4.1 min in GC–tandem MS). The longer retention times of the PFB derivatives in GC–tandem MS analyses is a result of the longer capillary column used, i.e., 30 m vs. 15 m.

3. Results

The most intense mass fragments in the mass spectrum of the PFB derivative of unlabeled nitrite were *m/z* 46 ($[NO_2]^-$, 100%), *m/z* 181 ($[PFB]^-$, 10%) and *m/z* 226 ($[PFB-NO_2-1]^-$, 40%). In the mass spectrum of the PFB derivative of ^{15}N-labeled nitrite the most intense mass fragments were *m/z* 47 ($[^{15}NO_2]^-$, 100%), *m/z* 181 ($[PFB]^-$, 10%) and *m/z* 227 ($[PFB-^{15}NO_2-1]^-$, 38%). CID of the parent ion of *m/z* 226 ($[M-1]^-$) of the PFB derivative of unlabeled nitrate resulted in the generation of daughter ions at *m/z* 147 (10%), *m/z* 112 (15%) and *m/z* 66 (100%). CID of the parent ion of *m/z* 227 ($[M-1]^-$) of the PFB derivative of ^{15}N-labeled nitrite resulted in the generation of daughter ions at *m/z* 147 (10%), *m/z* 113 (18%) and *m/z* 67 (100%). Surprisingly, CID of the parent ions of PFB derivatives of unlabeled and labeled nitrite did not result in formation of the daughter ions at *m/z* 46 and *m/z* 47, in contrast to the corresponding NTMB derivatives [3]. The structure of the most intense daughter ions at *m/z* 66 and *m/z* 67 observed from CID of

$[M-1]^-$ of the PFB derivatives of unlabeled and ^{15}N-labeled nitrite have not yet been identified.

GC–MS and GC–tandem MS analyses of nitrate (800 μM) yielded a peak area ratio of m/z 47 to 46 of 0.0054 and a peak area ratio of m/z 67 to 66 of 0.039. GC–MS and GC–tandem MS analyses of [^{15}N]nitrate (800 μM) resulted in a peak area ratio of m/z 46 to 47 of 0.017 and in a peak area ratio of m/z 66 to 67 of 0.016, respectively. Thus, both analyte and internal standard have ions at both mass fragments measured. In such cases the measured peak area ratio R of m/z 46 to m/z 47 from GC–MS analysis and of m/z 66 to m/z 67 from GC–tandem MS analysis in a sample can be expressed by the following equation [5]:

$$R = \{([NO_3^-]_s + [NO_3^-]_b) \times P_c + ([^{15}NO_3^-]_s \times Q_c)\}/\{([NO_3^-]_s + [NO_3^-]_b) \times P_{is} + ([^{15}NO_3]_s \times Q_{is})\} \quad (1)$$

where $[NO_3^-]_s$ and $[NO_3^-]_b$ are the concentrations of nitrate in the sample and in the buffer, respectively, $[^{15}NO_3^-]_s$ is the concentration of [^{15}N]nitrate in the sample or in the buffer, P_c is the relative intensity of the mass fragment m/z 46 or 66 originating from nitrate, P_{is} is the relative intensity of the mass fragment m/z 47 or 67 originating from nitrate, Q_{is} is the relative intensity of the mass fragment m/z 47 or 67 originating from [^{15}N]nitrate, and Q_c is the relative intensity of the mass fragment m/z 46 or 66 originating from [^{15}N]nitrate.

With the approximation that P_c and Q_{is} are both close to the unity Eq. (1) can be simplified to Eqs. (2a) and (2b):

$$R = \{([NO_3^-]_s + [NO_3^-]_b) + ([^{15}NO_3^-]_s \times Q_c)\}/\{([NO_3^-]_s + [NO_3^-]_b) \times P_{is} + [^{15}NO_3^-]_s\} \quad (2a)$$

$$R_b = ([NO_3^-]_b + [^{15}NO_3^-]_s \times Q_c)/([NO_3^-]_b \times P_{is} + [^{15}NO_3^-]_s) \quad (2b)$$

where R and R_b are the peak area ratios in the sample and in the buffer, respectively.

Standard curves were generated by GC–MS and GC–tandem MS for nitrate (0–4000 μM) in buffer using [^{15}N]nitrate at 800 μM. Fig. 1A shows that the peak area ratio of m/z 46 to 47 is linear in the whole range of the concentration ratio investigated. This suggests that Q_c and P_{is} can be neglected in GC–MS analyses, i.e., $Q_c = P_{is} \approx 0$. Under this assumption Eqs. (2a) and (2b) are transformed into the Eqs. (3a) and (3b), respectively.

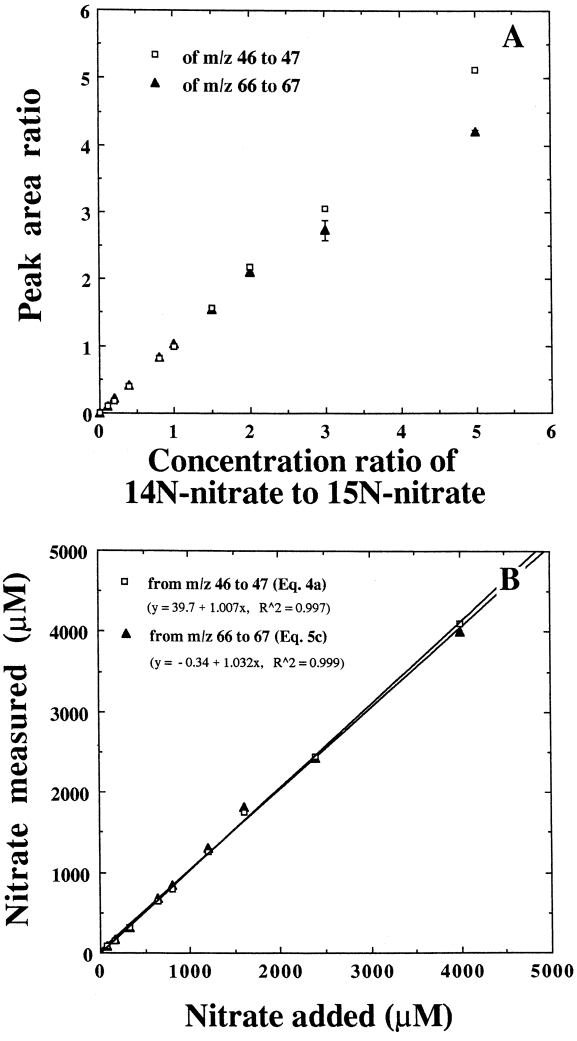

Fig. 1. (A) Peak area ratios (mean±SD) of m/z 46 to 47 (from GC–MS analysis) and of m/z 66 to 67 (from GC–tandem MS analysis) of nitrate in buffer using [^{15}N]nitrate at 800 μM. (B) Plot of nitrate concentrations measured by GC–MS and GC–tandem MS analyses. Nitrate concentrations from GC–MS analyses were calculated by using Eq. (4a) with $R_b = 0$. Nitrate concentrations from GC–tandem MS analyses were calculated using Eq. (5c). In both cases the mean values for R were used.

$$R = ([NO_3^-]_s + [NO_3^-]_b)/([^{15}NO_3^-]_s) \quad (3a)$$

$$R_b = [NO_3^-]_b/[^{15}NO_3^-]_s \quad (3b)$$

Combination of Eqs. (3a) and (3b) and resolving for $[NO_3^-]_s$ and $[NO_3^-]_b$ yields the Eqs. (4a) and (4b), respectively:

$$[NO_3^-]_s = [^{15}NO_3^-]_s \times (R - R_b) \quad (4a)$$

$$[NO_3^-]_b = [^{15}NO_3^-]_s \times R_b \quad (4b)$$

Thus, nitrate concentrations in a nitrate-containing biological sample and in an aqueous solution can be accurately calculated from the peak area of m/z 46 to m/z 47 measured by GC–MS by using Eqs. (4a) and (4b), respectively. Fig. 1B demonstrates the validity of Eq. (4b).

On the other hand, linearity between the peak area ratio of m/z 66 to 67 and the [^{14}N]nitrate/[^{15}N]nitrate ratio exists only up to [^{14}N]nitrate/[^{15}N]nitrate ratios of 1.0 (Fig. 1A). Above this ratio the peak area ratio of m/z 66 to 67 becomes hyperbolic suggesting that Eqs. (4a) and (4b) cannot be used in GC–tandem MS to accurately calculate nitrate concentrations exceeding those of the internal standard. Combination of Eqs. (2a) and (2b) and resolving for $[NO_3^-]_s$ reveals the following expression:

$$[NO_3^-]_s = [^{15}NO_3^-]_s\{(R - Q_c)/(1 - P_{is} \times R) - (R_b - Q_c)/(1 - P_{is} \times R_b)\} \quad (5a)$$

Setting into Eq. (5a) the measured values for Q_c and P_{is} of 0.016 and 0.039 in GC–tandem MS analyses, respectively, yields Eq. (5b):

$$[NO_3^-]_s = [^{15}NO_3^-]_s \times \{(R - 0.016)/(1 - 0.039 \times R) - (R_b - 0.016)/(1 - 0.039 \times R_b\} \quad (5b)$$

Typical R_b and R values from GC–tandem MS analyses in human plasma samples are of the order of 0.5 and 1.0–1.5, respectively. Typical R_b and R from GC–tandem MS analyses in human urine samples are of the order of 0.04 and 0.5–2.0, respectively.

For aqueous solutions in which the nitrate levels in the buffer are negligible in comparison with $[^{15}NO_3^-]_s$, i.e., $R \gg R_b$, Eq. (5b) is simplified to Eq. (5c):

$$[NO_3^-]_s = [^{15}NO_3^-]_s \times \{(R - 0.016)/(1 - 0.039 \times R)\} \quad (5c)$$

Using Eq. (5c) we calculated nitrate concentrations in aqueous buffer from the measured ratio R, i.e., m/z 66 to m/z 67, and plotted these concentrations vs. the nitrate concentrations (Fig. 1B). The good agreement between nitrate concentrations measured by GC–MS and GC–tandem MS and the almost identical slopes of the standards curves which are close to unity indicate that nitrate can be accurately determined in aqueous buffer by GC–tandem MS utilizing Eq. (5c).

A series of human plasma and urine samples were analyzed in duplicate by GC–MS by selected ion monitoring of m/z 46 for nitrite and m/z 47 for [^{15}N]nitrite and by GC–tandem MS by selected reaction monitoring of m/z 66 for nitrite and m/z 67 for [^{15}N]nitrite. Nitrate concentrations from GC–MS and GC–tandem MS analyses were calculated from the respective peak area ratios measured applying Eq. (4a) and Eq. (5b), respectively. Representative chromatograms from GC–MS and GC–tandem MS analyses of nitrate in a plasma and in a urine sample of a healthy volunteer are shown in Fig. 2. In plasma samples of 28 humans nitrate was determined from duplicate analyses as (mean±S.D.) 25.6±8.7 μM by GC–MS and 26.7±8.9 μM by GC–tandem MS. In urine samples of ten humans nitrate was determined as 518±728 μM by GC–MS and 524±713 μM by GC–tandem MS. The coefficient of correlation between GC–tandem MS and GC–MS was 0.985 for plasma and 0.992 for urine samples. Mean RSD was below 4% for plasma and urine samples using both GC–MS and GC–tandem MS. Instrumental precision from repeated analysis (each $n=7$) of the same plasma sample was 1.3% by GC–MS and 1.5% by GC–tandem MS.

Also, a series of 20 human urine samples alongside with three quality control samples were analyzed by GC–MS using both instruments and by GC–tandem MS using the TSQ instrument. For quality control a pooled urine sample of a healthy volunteer was used. Six 1-ml aliquots of the urine were spiked each with 800 μM of [^{15}N]nitrate. Two

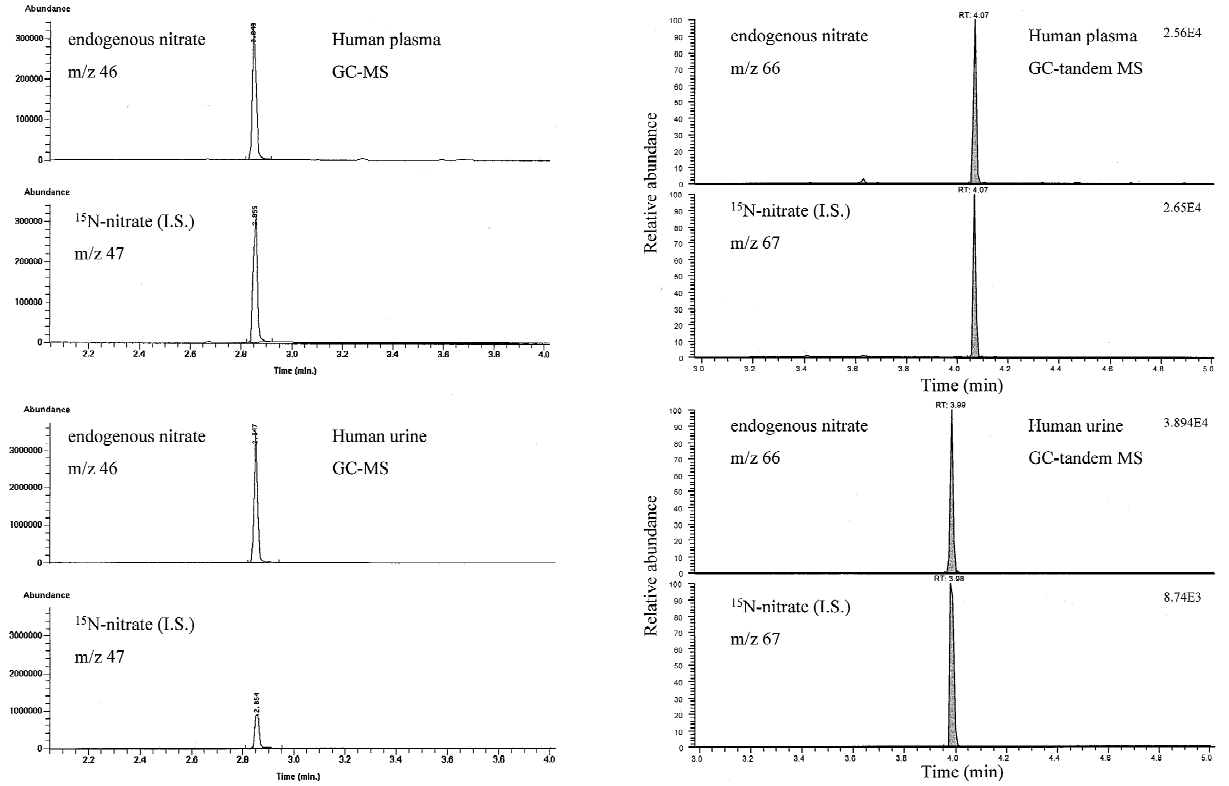

Fig. 2. Typical chromatograms from the GC–MS (left panel) and GC–tandem MS (right panel) analysis of nitrate in human plasma (upper traces) and human urine (lower traces). [^{15}N]Nitrate was used as internal standard at 40 μM for the plasma and 800 μM for the urine sample. The smaller retention time of the PFB derivative in urine samples resulted from the shortening of the capillary column of the GC–tandem MS instrument.

1-ml aliquots of them were not spiked with nitrate (quality control sample 1, QC1), another two 1-ml aliquots were spiked with 400 μM of nitrate (quality control sample 2, QC2), and finally two 1-ml aliquots were spiked with 800 μM of nitrate (quality control sample 3, QC3). The results of the measurement of these samples are summarized in Table 1. Accuracy for QC2 and QC3 were 100 and 99% for the HP instrument, 105 and 95% and 96 and 97% for the TSQ instrument in the GC–MS and GC–tandem MS mode, respectively. Precision (RSD) was above 96% in all quality control samples for both instruments.

The limits of detection (each at a S/N of 3:1) from analyses of aqueous solutions of nitrite were determined as 20 amol of nitrite by GC–MS and 800 amol of nitrite by GC–tandem MS (TSQ instrument) at RSD values of 8% and 12%, respectively. The considerably higher limit of detection for nitrite in GC–tandem MS results from the use of a less intense ion for CID and from a low ion transmission and low collision efficiency.

4. Discussion

In the present study we investigated the utility of GC–tandem MS for the quantitative determination of nitrate in human plasma and urine samples. Surprisingly, and in contrast to the NTMB derivative of nitrate [3], CID of the parent ions $[M-1]^-$ of PFB derivatives of unlabeled and ^{15}N-labeled nitrite generated intense daughter ions at m/z 66 and m/z 67 but not at m/z 46 and m/z 47. The ions with m/z 46 and m/z 47 correspond to the anions NO_2^- and $^{15}NO_2^-$, respectively. Because of the relatively low

Table 1
Nitrate levels in urine of 20 healthy volunteers as measured by GC–MS performed on two instruments and by GC–tandem MS carried out on the TSQ instrument[a]

Urine sample number	Nitrate (μM) measured by GC–MS (HP)	Concentration ratio of HP to TSQ	Nitrate (μM) measured by GC–MS (TSQ)	Concentration ratio of GC–MS to GC–tandem MS	Nitrate (μM) measured by GC–tandem MS (TSQ)
1	966	1.0189	948	0.9854	962
2	436	1.0000	436	0.9819	444
3	4349	1.0142	4288	0.9777	4386
4	414	0.9741	425	1.0241	415
5	989	1.0010	988	0.9979	990
6	727	0.9918	733	0.9799	748
7	1133	1.0171	1114	0.9832	1133
8	957	1.0010	956	0.9866	969
9	311	0.9904	314	0.9968	315
10	709	1.0143	699	0.9859	709
11	2739	0.9838	2784	0.9636	2889
12	428	1.0166	421	0.9906	425
13	282	0.9758	289	0.9698	298
14	930	0.9862	943	0.9761	966
15	737	0.9814	751	1.0287	730
16	700	1.0000	700	0.9589	735
17	2572	0.9680	2657	1.0615	2503
18	622	1.0032	620	0.9657	642
19	726	1.0083	720	0.9511	757
20	166	0.9881	168	0.9438	178
Mean±SD (RSD)		0.9967±0.0155 (1.56%)		0.9855±0.0275 (2.79%)	
QC1	381	0.9948	383	0.9647	397
QC2	781	0.9714	804	1.0281	782
QC3	1172	1.0227	1146	0.9786	1171

[a] HP = Hewlett Packard; TSQ = Thermoquest. The ratios of the values between nitrate levels measured by HP and TSQ and between those measured by TSQ in the MS and tandem MS mode are also given. Urinary nitrate levels from GC–MS and GC–tandem MS analyses were calculated by using Eq. (4a) and Eq. (5b), respectively.

natural abundance of ^{15}N and ^{17}O the contribution of m/z 47 originating from the ^{15}N-isotope of NO_2^- to m/z 47 of the internal standard is negligible. The finding from GC–tandem MS analyses that m/z 67 originating from the ^{15}N-isotope of nitrate contributes significantly to m/z 67 of the internal standard, i.e., [^{15}N]nitrate, suggests that the daughter ions at m/z 66 and m/z 67 may contain up to three C atoms the ^{13}C-isotope of which curves the calibration at concentrations of nitrate exceeding those of [^{15}N]nitrate [5]. Additional experiments are necessary to elucidate the structure of the daughter ions with m/z 66 and 67.

Nitrate concentrations in plasma and urine measured by GC–MS can be easily calculated using Eq. (4a) by multiplying the difference of the measured peak area ratios in the sample and the buffer, i.e., $(R-R_b)$, with the concentration of the internal standard, i.e., [$^{15}NO_3^-$]. On the hand, because the daughter ions at m/z 66 and m/z 67 have considerably more abundant ions at both mass fragments, no linear relationship exists between the peak area ratio of m/z 66 to 67 and nitrate/[^{15}N]nitrate concentration ratios above 1.0. For the calculation of any nitrate concentration in a biological sample from GC–tandem MS analyses we derived and applied successfully Eq. (5b). Plasma and urine nitrate concentrations calculated by this equation are very close to those from GC–MS analyses underlying the validity of Eq. (5b). On the basis of the generally higher specificity of GC–tandem MS our study verifies our previously reported GC–MS method that measures nitrate — after its reduction to nitrite — and nitrite as PFB derivatives [2] and proves plasma and urine nitrate levels measured by us previously by using GC–MS to be true [2,4]. Quantification of

plasma and urinary nitrate as PFB derivative by GC–MS is several times more sensitive than by GC–tandem MS and possesses a comparable specificity. Therefore, quantification of nitrate as PFB derivative is satisfactory accomplished by GC–MS. However, GC–tandem MS of nitrate as PFB derivative is an additional valuable analytical tool for the verification of nitrate levels measured by GC–MS and other techniques in complex biological fluids.

Determination of nitrate by GC–MS as PFB derivative requires reduction of nitrate to nitrite prior to derivatization because nitrate does not react with PFB bromide. The results of the present work and of two previously reports of our group [2,4] show that reduction of nitrate to nitrite is not crucial in GC–MS and GC–tandem MS methods. With the exception of the exogenous N^G-nitroarginine that contributes to nitrate when reduction is performed by cadmium [6] we did not find any interference in our method. By contrast, Rhodes et al. did find considerable interference by nonfree-nitrite/nitrate sources in human urine and plasma when measured by a GC–MS method that involves concentrated sulfuric acid-catalysed conversion of nitrate to nitrobenzene [7]. Moreover, nitrite, which occurs in human plasma and urine at concentrations much lower than those of nitrate, can be determined specifically, i.e., in the present of high excess of nitrate, by GC–MS or GC–tandem MS as its PFB derivative without any other treatment except for the derivatization. By contrast, quantification of nitrite in human plasma and urine as NTMB or nitrobenzene by GC–MS or GC–tandem MS requires two separate derivatization steps for the determination of nitrate and of the sum of nitrate plus nitrite. The concentration of nitrite is then calculated by difference, i.e., by subtracting the concentration of nitrate from that of the sum of nitrate plus nitrite. Because the concentration of nitrate amounts to more than 95% of the sum of nitrate plus nitrite in human plasma and about 100% in human urine [2] quantification of nitrite by GC–MS as NTMB or nitrobenzene would be inaccurate in plasma and even impossible in urine of humans. Thus, GC–MS analysis of nitrite and nitrate-derived nitrite as PFB derivative is currently the most reliable approach to quantitate these anions accurately and sensitively in various biological fluids.

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