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Journal of Chromatography B, 784 (2003) 77–90
JOURNAL OF CHROMATOGRAPHY B
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Accurate quantification of basal plasma levels of 3-nitrotyrosine and 3-nitrotyrosinoalbumin by gas chromatography–tandem mass spectrometry

Dimitrios Tsikas*, Edzard Schwedhelm[1], Friedericke K. Stutzer, Frank-Mathias Gutzki, Iris Rode, Christina Mehls, Jürgen C. Frölich

Institute of Clinical Pharmacology, Hannover Medical School, Carl-Neuberg-Strasse 1, 30625 Hannover, Germany

Received 30 July 2002; received in revised form 17 September 2002; accepted 23 September 2002

Abstract

Measurement of 3-nitro-L-tyrosine (NO_2Tyr) and protein-related 3-nitro-L-tyrosine in human plasma is associated with numerous methodological problems which result in highly divergent basal plasma levels often ranging within two orders of magnitude. Recently, we have described an interference-free GC–tandem MS-based method for NO_2Tyr which yielded the lowest basal plasma NO_2Tyr levels reported thus far. This method was extended to quantify protein-associated 3-nitrotyrosine and in particular 3-nitrotyrosinated albumin (NO_2TyrALB) in human plasma. NO_2TyrALB and albumin (ALB) were extracted from plasma by affinity column extraction and digested enzymatically at neutral pH. 3-Nitro- L-[$^2H_3$]tyrosine was used as internal standard. In plasma of 18 healthy young volunteers the molar ratio of NO_2TyrALB to albumin-derived tyrosine (TyrALB), i.e. NO_2TyrALB/TyrALB, was determined to be $1.55\pm0.54\times1{:}10^6$ (mean±SD). The plasma concentration of NO_2TyrALB was estimated as 24±4 n*M*. The NO_2Tyr plasma levels in these volunteers were determined to be 0.73±0.53 n*M*. In the same volunteers, NO_2TyrALB/TyrALB, NO_2TyrALB and NO_2Tyr were measured 15 days later and the corresponding values were determined to be $1.25\pm0.58\times1{:}10^6$, 25±6 n*M* and 0.69±0.16 n*M*. For comparison, NO_2Tyr and NO_2TyrALB were measured in six plasma samples from healthy volunteers by GC–MS and GC–tandem MS. Different values were found for NO_2Tyr, i.e. 5.4±2.8 versus 2.7±1.5 n*M*, and comparable values for NO_2TyrALB/TyrALB, i.e. $0.5\pm0.2\times1{:}10^6$ versus $0.4\pm0.1\times1{:}10^6$, by these methods. The ratio of the values measured by GC–MS to those measured by GC–tandem MS were 2.9±3.1 for NO_2Tyr and 1.2±0.2 for NO_2TyrALB/TyrALB. The present GC–tandem MS method provides accurate values of NO_2Tyr and NO_2TyrALB in human plasma.

Keywords: 3-Nitrotyrosine; 3-Nitrotyrosinoalbumin

1. Introduction

Reactive-nitrogen species (RNS) such as nitrogen monoxide (NO) and nitrogen dioxide (NO_2), peroxynitrite ($ONOO^-$) and nitryl chloride (NO_2Cl) react readily with tyrosine (Tyr) and protein-associated tyrosine (TyrProt) to form 3-nitrotyrosine, i.e.

*Corresponding author. Tel.: +49-511-532-3959; fax: +49-511-532-2750.
E-mail address: tsikas.dimitros@mh-hannover.de (D. Tsikas).
[1]Present address: Institute of Experimental and Clinical Pharmacology, University Hospital Hamburg-Eppendorf, Germany.

1570-0232/02/$ – see front matter
PII: S1570-0232(02)00751-1

NO_2Tyr and NO_2TyrProt, respectively. Therefore, detection of NO_2Tyr and/or NO_2TyrProt provides evidence for generation of RNS [1]. Besides the uncertainty of what actually nitrates tyrosine, the measurement of NO_2Tyr and NO_2TyrProt in biological samples, especially in human plasma, is associated with many methodological problems the most serious of which include artifactual formation and lack of sensitivity and specificity.

Artifactual formation of NO_2Tyr and NO_2TyrProt from tyrosine and nitrate and/or nitrite occurs from acidification of biological samples [2–10]. It has been shown by many groups that avoidance of acidic conditions during sample treatment minimizes artifactual formation of 3-nitrotyrosine [3–6,9]. Regarding NO_2TyrProt, this means that plasma proteins must be hydrolyzed under non-acidic conditions, preferably enzymatically [6]. Especially in mass spectrometry (MS)-based methods for basal 3-nitrotyrosine, in which artifactual formation of 3-nitrotyrosine from the indispensable derivatisation procedure(s) may occur [3,4,9,10], separation of NO_2Tyr from Tyr is absolutely required and can be easily achieved by HPLC [9].

Several reported methods such as HPLC and even LC–tandem MS lack sufficient sensitivity to detect basal plasma levels of NO_2Tyr [3,11]. Also, denaturation of plasma proteins may lead to overestimated NO_2Tyr levels. This can be avoided by using mild conditions for plasma generation, immediate analysis of the plasma ultrafiltrate samples and by thawing plasma samples stored at −80 °C once only [9]. Presumably, these difficulties have led to highly divergent values for NO_2Tyr and NO_2TyrProt in human plasma at the basal state and in rat plasma [3–12].

Due to the potential importance of NO_2TyrProt such as 3-nitrotyrosinated albumin (NO_2TyrALB) as quantitative biomarkers of RNS-induced oxidative stress in the human circulation, we extended our GC–tandem MS-based method originally developed for plasma NO_2Tyr [9] to quantify NO_2TyrALB in human plasma. Considering all the known and potential sources for artifacts we have developed a method that allows highly specific, interference-free and accurate quantitative determination of NO_2TyrALB in human plasma by GC–tandem MS. The NO_2TyrALB and NO_2Tyr levels measured in plasma of healthy humans at the basal state by the present GC–tandem MS method are the lowest so far reported for 3-nitrotyrosinated plasma proteins.

2. Experimental

2.1. Materials and chemicals

3-Nitro-L-tyrosine, L-tyrosine, L-phenylalanine, *p*-nitro-L-phenylalanine and bovine serum albumin (BSA) were purchased from Sigma (Deisenhofen, Germany). Tetramethylammonium peroxynitrite was bought from ALEXIS (Grünberg, Germany). 3-Nitro-L-[$^2H_3$]tyrosine (d_3-NO_2Tyr; 98 atom% at ^2H) was synthesized as described elsewhere [9]. Pentafluoropropionic anhydride and *N,O*-bis-(trimethylsilyl)trifluoroacetamide were obtained from Pierce (Rockford, IL, USA). Centrisart I ultrafiltration cartridges (pore size 4 μm, cut-off 20 kDa) were supplied by Sartorius (Göttingen, Germany). Chromabond HR-P solid-phase extraction cartridges (1 ml, 100 mg) were purchased from Macherey-Nagel (Düren, Germany). HiTrapBlue Sepharose affinity columns (1 ml) were obtained from Pharmacia Biotech (Freiburg, Germany). All other chemicals were obtained from Merck (Darmstadt, Germany). Pronase from *Streptomyces griseus* was purchased from Boehringer Mannheim (Mannheim, Germany). This enzyme preparation (declared specific activity: 7 μmol tyrosine/min×mg) consisted of various unspecific proteolytic enzymes with molecular masses varying between 20 and 60 kDa. Solutions of pronase in distilled water with a content of 20 mg/ml or in buffer with a content of 6 mg/ml were freshly prepared.

2.2. Sample preparation procedures

Blood (5 ml) was drawn from antecubital veins of healthy volunteers using syringes containing EDTA and put immediately on ice. Blood samples were centrifuged at 1500 *g* and 2 °C for 15 min. The plasma generated was used immediately or stored at −80 °C until further analysis. Plasma NO_2Tyr was determined exactly as described previously [9]. ALB and NO_2TyrALB were extracted from plasma by affinity column chromatography as described previ-

ously for *S*-nitrosoalbumin [13]. Briefly, 0.4-ml aliquots of plasma were applied to a 1-ml HiTrap-Blue Sepharose affinity column preconditioned with 4 ml of buffer A (50 m*M* KH_2PO_4, pH 7.0). Cartridges were washed with 4 ml of buffer A and proteins were eluted with 2 ml of buffer B (50 m*M* KH_2PO_4, 1.5 *M* KCl, pH 7.0). ALB and NO_2TyrALB were proteolysed directly in the eluate (1 ml) by adding 1 mg of pronase and by incubating the mixture at 37 °C for 20 h. After addition of 9.8 pmol of d_3-NO_2Tyr the sample was ultrafiltered (1500 *g*, 4 °C, 15 min) and the ultrafiltrate was stored at −20 °C until further analysis. Under similar conditions tyrosine and phenylalanine were obtained from commercially available HSA (20 mg) digested by pronase (4 mg) in buffer B with a recovery of 54±8 and 52±10% (mean±SD, $n=4$) as found by HPLC (see below).

3-Nitrotyrosinated plasma proteins (i.e. NO_2TyrProt) were determined in the protein fraction of ultrafiltered plasma samples (2 ml) from centrifugation (1500 *g*, 4 °C, 4 h). The protein fraction was diluted with distilled water to obtain a final protein concentration of 160 mg/ml as measured by the Lowry assay. To a 125-μl aliquot of the resulting solution were added a 200-μl aliquot of the pronase solution in distilled water (20 mg protein/ml) and a 500-μl aliquot of 0.2 *M* Tris buffer, pH 7.5, and the mixture was incubated at 37 °C for 20 h. After proteolysis the mixture was spiked with 9.8 pmol of d_3-NO_2Tyr and ultrafiltered by centrifugation (1500 *g*, 4 °C, 20 min).

Aliquots (200 μl) of ultrafiltrate samples from native plasma or from proteolysed samples were analysed by HPLC as described [9]. *p*-Nitro-L-phenylalanine coelutes with NO_2Tyr in this HPLC system and was used to determine the retention time (t_R) of NO_2Tyr. The HPLC fraction (3 ml) eluting with the t_R of NO_2Tyr was collected and compounds were isolated by solid-phase extraction (SPE) on HR-P cartridges [9]. After solvent evaporation under a stream of nitrogen, amino acids were converted into their *n*-propyl ester-pentafluoropropionyl amide-trimethylsilyl ether derivatives exactly as described [9].

Tyr and L-phenylalanine (Phe) obtained from proteolysis of plasma proteins were quantitated by the same HPLC system [9] by ultraviolet absorbance detection at 276 nm for Tyr and 236 nm for Phe. Ultrafiltered proteolysed mixtures (10 μl) were diluted with the mobile phase (1:100, v/v), and 100-μl aliquots of dilutions were injected. Standard curves of authentic Tyr (t_R 2.3 min) and Phe (t_R 6.7 min) in buffer B (range 0–100 μ*M* each) were used for calibration.

2.3. Gas chromatography–tandem mass spectrometry

GC–MS and GC–tandem MS analyses were performed in the electron capture negative-ion chemical ionisation mode on a triple-stage quadrupole mass spectrometer ThermoQuest TSQ 7000 (Finnigan MAT, San Jose, CA) directly interfaced with a Trace 2000 series gas chromatograph equipped with an autosampler AS 2000 (CE Instruments, Austin, TX). The gas chromatograph was equipped with a fused-silica capillary column Optima 5-MS (30 m×0.25 mm I.D., 0.25-μm film thickness) from Macherey-Nagel (Düren, Germany). The following oven temperature program was used with helium (at a constant pressure of 55 kPa) as the carrier gas: 2 min at 80 °C, then increased to 320 °C at a rate of 25 °C/min and kept at 320 °C for 5 min. Interface, injector and ion source were kept at 280, 280 and 180 °C, respectively. Electron energy and electron current were set to 200 eV and 600 μA, respectively. Methane (530 Pa) and argon (0.13-Pa collision pressure) were used as reagent and collision gases, respectively. Collision energy was set to 6 eV. Electron multiplier voltage was set to 1–2 kV. Aliquots (1 μl) were injected in the splitless mode by the autosampler.

Quantification by GC–MS was performed by selected ion monitoring (SIM) of the ions at m/z 396 and 399 for the *n*-propyl ester-pentafluoropropionyl amide-trimethylsilyl ether derivatives (i.e. *n*-propyl-PFP-TMS) of NO_2Tyr and d_3-NO_2Tyr, respectively. Quantification by GC–tandem MS was performed by selected reaction monitoring (SRM) of the product ions at m/z 379 and 382 which were obtained by collision-activated dissociation (CAD) of the parent ions at m/z 396 and 399, respectively [9].

Data are presented as mean±SD from at least duplicate analyses if not otherwise specified. The significance of differences was determined with the paired *t*-test; $P<0.05$ was considered significant.

3. Results

3.1. Nitration of tyrosine residues in bovine serum albumin by peroxynitrite and utility of d_3-NO_2Tyr as internal standard for 3-nitrotyrosinated proteins

Treatment of BSA (604 μ*M*) with authentic peroxynitrite (up to 300 μ*M*) at physiological pH resulted in a concentration-dependent formation of 3-nitrotyrosinated BSA (NO_2TyrBSA) (Fig. 1). The measured molar ratio of NO_2TyrBSA to BSA-associated Tyr (TyrBSA) using 300 μ*M* of peroxynitrite amounted to $0.58\times1{:}10^3$. Quantitative nitration of TyrBSA (11 476 μ*M*, i.e. 604 μ*M*×19 Tyr molecules per BSA molecule [14]) by 300 μ*M* peroxynitrite would yield 300 μ*M* of NO_2TyrBSA, i.e. a molar ratio of $26.8\times1{:}10^3$ for NO_2TyrBSA/TyrBSA. Thus, the extent of nitration of TyrBSA by 300 μ*M* of peroxynitrite is calculated to be ~2%. Under similar experimental conditions 300 μ*M* of peroxynitrite reacted with 1000 μ*M* of Tyr to form 12 μ*M* of NO_2Tyr equivalent to a yield of ~4% with respect to peroxynitrite.

NO_2TyrBSA prepared by nitration of BSA (604 μ*M*) with peroxynitrite (300 μ*M*) was used to test the applicability of d_3-NO_2Tyr as an internal standard for the measurement of protein-associated 3-nitrotyrosine by GC–tandem MS. The linear relationship between the peak area ratio of m/z 379 to m/z 382 (y) and the peroxynitrite-treated BSA amount added to 20 mg of plasma proteins (x) obtained with the regression equation $y = 0.217 + 2.59\times10^{-3}x$, $r = 0.9979$, confirms the utility of d_3-NO_2Tyr as internal standard (Fig. 2). Assuming that the plasma proteins used have a mean molecular weight equivalent to that of human serum albumin, i.e. 68 000, and that the extent of proteolysis was 50%, the y-axis intercept (y_0) allows estimation of the 3-nitrotyrosine amount present in 20 mg (i.e. 294 nmol) of plasma proteins. It results from this that $NO_2\text{Tyr} = 2\times y_0 \times d_3\text{-}NO_2\text{Tyr} = 2\times0.217\times10$ pmol $= 4.34$ pmol, with the factor 2 considering the proteolysis recovery of 50% or 0.5. Thus, 1 mol of plasma proteins would contain ~14.8 μmol of NO_2Tyr (i.e. 4.34 pmol/294 nmol). This means that the concentration of 3-nitrotyrosinated proteins in human plasma would amount to a few nM.

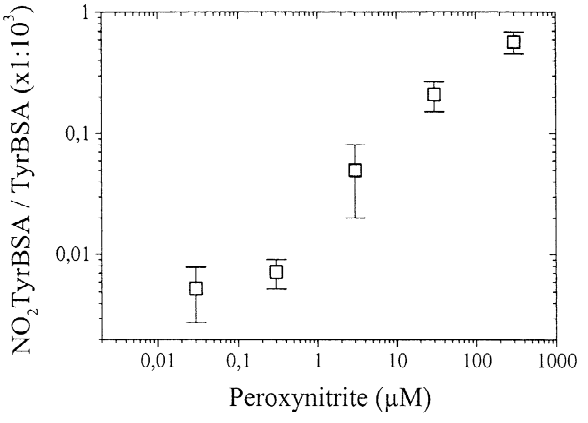

Fig. 1. 3-Nitrotyrosination of bovine serum albumin (BSA) by peroxynitrite in phosphate buffer. Solutions (40 mg/ml, 604 μ*M*) of BSA in 0.1 *M* sodium phosphate buffer, pH 7.4, containing 0.1 *M* DTPA (diethyltriaminepentaacetate), were treated with peroxynitrite at final concentrations of 0, 0.3, 3, 30 and 300 μ*M*, and incubated at 25 °C for 1 h. 3-Nitrotyrosine from proteolysed NO_2TyrBSA was determined by GC–tandem MS; L-tyrosine from proteolysed TyrBSA/NO_2TyrBSA was determined by HPLC. Data are shown as mean±SD from duplicate incubates.

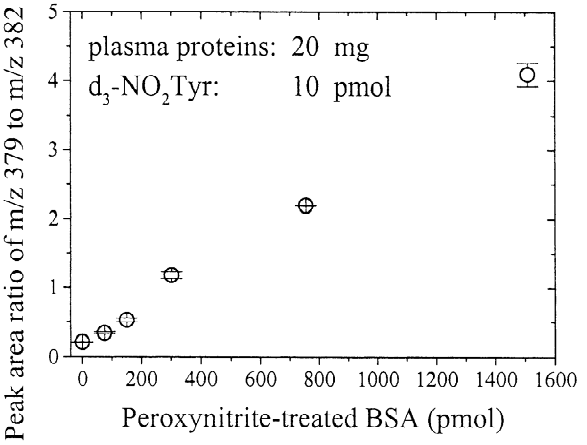

Fig. 2. GC–tandem MS standard curve for 3-nitrotyrosinoproteins in human plasma. Each 20 mg of human plasma proteins, which were obtained by ultrafiltration of pooled human plasma, were spiked with varying amounts of peroxynitrite-nitrated bovine serum albumin (NO_2TyrBSA) as indicated. Samples were spiked with 10 pmol of the internal standard (I.S.) 3-nitro-L-[$^2H_3$]tyrosine (d_3-NO_2Tyr) and analysed by GC–tandem MS in the SRM mode after enzymic proteolysis, HPLC analysis and derivatization as described in the Experimental section. Data are shown as mean±SD from duplicate incubates.

Table 1
Comparison of GC–MS with GC–tandem MS regarding NO_2Tyr levels in plasma of 12 healthy volunteers (aged 51±10 years)

Plasma no.	NO_2Tyr (n*M*)		
	GC–MS[a]	GC–tandem MS[a]	MS to MS–MS
1	0.94	0.75	1.247
2	3.87	2.16	1.792
3	0.75	0.66	1.137
4	1.18	1.52	0.776
5	0.62	0.70	0.886
6	5.03	0.60	8.383
7	5.03	0.39	12.89
8	5.05	0.63	8.008
9	0.99	1.00	0.990
10	4.19	0.67	6.293
11	10.4	2.24	4.643
12	15.5	2.36	6.301
Mean±SD	4.463±4.495	1.140±0.727	4.446±3.962

NO_2Tyr was determined in plasma ultrafiltrate samples.
[a] $P = 0.0166$ for NO_2Tyr between GC–MS and GC–tandem MS, *t*-test.

3.2. Basal plasma levels of free 3-nitrotyrosine by GC–MS and GC–tandem MS

In plasma samples of healthy volunteers NO_2Tyr was determined both by GC–MS and GC–tandem MS (Tables 1 and 2). For comparison the ratio of the concentrations measured by GC–MS to those measured by GC–tandem MS was calculated. GC–MS and GC–tandem MS were compared on an individual basis. NO_2Tyr was measured in plasma of a collective of 12 healthy elderly volunteers by GC–MS and GC–tandem MS (Table 1). Plasma levels of NO_2Tyr in these subjects were measured as 4.46±4.49 n*M* by GC–MS and as 1.14±0.73 n*M* by GC–tandem MS (Table 1). The ratio of the NO_2Tyr values obtained from GC–MS to those obtained from GC–tandem MS was 4.45±3.96. The difference between the GC–MS and GC–tandem MS values for NO_2Tyr was statistically significant ($P = 0.02$). In a second collective of six healthy young volunteers no

Table 2
Comparison of GC–MS with GC–tandem MS regarding measurement of NO_2TyrALB and NO_2Tyr in plasma of six healthy volunteers (aged 25±3 years)

Plasma sample	NO_2Tyr (n*M*)			NO_2TyrALB/TyrALB ($1:10^6$)		
	GC–MS[a]	GC–tandem MS[a]	MS to MS–MS	GC–MS[a]	GC–tandem MS[a]	MS to MS–MS
A	3.65	2.56	1.426	0.519	0.426	1.218
B	7.96	2.79	2.853	0.466	0.475	0.981
C	2.57	2.01	1.279	0.563	0.455	1.237
D	9.24	1.01	9.149	0.784	0.472	1.661
E	6.24	5.56	1.122	0.423	0.398	1.063
F	3.02	2.13	1.418	0.296	0.288	1.028
Mean±SD	5.447±2.783	2.677±1.540	2.875±3.137	0.509±0.163	0.419±0.071	1.198±0.249

NO_2Tyr was determined in plasma ultrafiltrate samples. NO_2TyrALB and TyrALB (by HPLC) were determined after affinity column extraction on HiTrapBlue Sepharose cartridges.
[a] $P = 0.087$ for NO_2Tyr, and $P = 0.124$ for NO_2TyrALB/TyrALB between GC–MS and GC–tandem MS, *t*-test.

correlation ($r = -0.014$) was found for plasma levels of NO_2Tyr measured by GC–MS (5.45±2.78 n*M*) and GC–tandem MS (2.67±1.54 n*M*) ($P = 0.12$; Table 2). The ratio of the NO_2Tyr values obtained from GC–MS to those obtained from GC–tandem MS was 2.87±3.14 in these subjects, suggesting considerable interference in the GC–MS analysis by coeluting substance(s).

3.3. Basal plasma ratios of 3-nitrotyrosinoalbumin to tyrosinoalbumin by GC–MS and GC–tandem MS

In previous work we have described a fully validated GC–tandem MS method for the quantitative determination of free NO_2Tyr in human plasma at the basal state [9]. Because of the lack of structurally entirely characterized synthetic standards for 3-nitrotyrosinated plasma proteins we were not able in the present study to determine the accuracy of the method for protein-associated 3-nitrotyrosine. The present method involves essentially the same analytical steps with the exception of the previously thoroughly investigated affinity column extraction for albumin [13,15] and the enzymic digestion which allows normalization of NO_2TyrALB by TyrALB. We, therefore, believe that the present method is as accurate and precise as the recently reported method for NO_2Tyr [9].

In plasma samples of healthy volunteers the concentration of NO_2TyrALB was determined both by GC–MS and GC–tandem MS (Table 2). For comparison the ratio of the concentrations measured by GC–MS to those measured by GC–tandem MS was calculated. GC–MS and GC–tandem MS were compared on an individual basis. NO_2TyrALB/TyrALB levels measured by GC–MS ($0.51 \pm 0.16 \times 1{:}10^6$) correlated ($r = 0.758$) with those measured by GC–tandem MS ($0.42 \pm 0.07 \times 1{:}10^6$). The ratio of the NO_2TyrALB/TyrALB values obtained from GC–MS to those obtained from GC–tandem MS was 1.198±0.25. The difference between the GC–MS and GC–tandem MS values for NO_2Tyr and NO_2TyrALB/TyrALB was statistically not significant ($P = 0.087$ and $P = 0.124$, respectively). These findings might suggest that GC–MS may be used for quantification of NO_2TyrALB in human plasma.

NO_2TyrALB, TyrALB, albumin-associated phenylalanine (PheALB) and NO_2Tyr were determined in freshly obtained plasma from venous blood taken from 18 healthy male volunteers (26±3 years) on 2 days at an interval of 2 weeks (i.e. day 1 and day 15) by GC–tandem MS (NO_2TyrALB and NO_2Tyr) and HPLC (TyrALB and PheALB) as described in the Experimental section. NO_2TyrALB/TyrALB, NO_2Tyr and TyrALB/PheALB levels were similar on both days (Fig. 3). The molar ratio of NO_2TyrALB/TyrALB amounted to $1.55 \pm 0.54 \times 1{:}10^6$ on day 1 and $1.25 \pm 0.58 \times 1{:}10^6$ on day 15. NO_2Tyr plasma levels amounted to 0.73±0.53 n*M* on day 1 and 0.69±0.16 n*M* on day 15. Also, very similar values were obtained for the molar ratio of TyrALB/PheALB on both days, i.e. 0.72±0.03 on day 1 versus 0.71±0.05 on day 15. The TyrALB/PheALB molar ratio measured is close to the theoretical value of 0.58 which is calculated by dividing the number of L-tyrosine moieties by the number of L-phenylalanine moieties in the whole human serum albumin molecule, i.e. 18:31 [14]. This close agreement and the finding that HiTrapBlue Sepharose affinity column extraction yields plasma albumin with a purity of 90% with respect to total plasma proteins [15] suggest that most L-tyrosine and L-phenylalanine measured originates from plasma albumin.

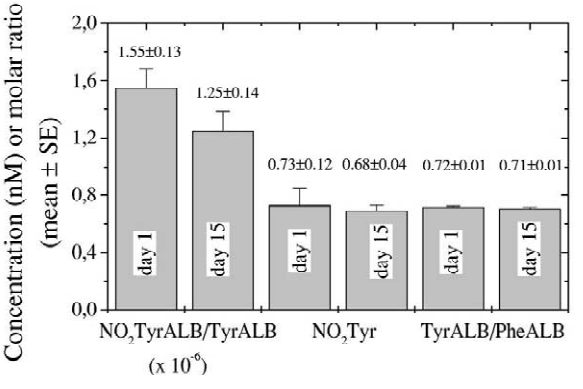

Fig. 3. Molar ratios of NO_2TyrALB/TyrALB and TyrALB/PheALB, and NO_2Tyr concentrations in venous blood plasma from 18 healthy young volunteers obtained on 2 different days at an interval of 2 weeks (i.e. day 1 and day 15). NO_2TyrALB and NO_2Tyr were determined by GC–tandem MS. TyrALB and PheALB were determined by HPLC.

The precision (RSD) of the method was determined by analysing in quadruplicate freshly obtained human plasma for NO_2TyrALB and NO_2Tyr. The molar ratios of NO_2TyrALB/TyrALB and TyrALB/PheALB and the plasma level of NO_2Tyr were measured as $0.975\pm0.096\times1{:}10^6$ (RSD, 9.8%), 0.593 ± 0.017 (RSD, 2.9%) and 0.820 ± 0.059 n*M* (RSD, 7.2%), respectively.

3.4. Basal plasma ratios of total 3-nitrotyrosinoproteins to tyrosinoproteins

In nine out of 12 elderly persons (Table 1) we also measured the ratio of total 3-nitrotyrosinoproteins to tyrosinoproteins, i.e. NO_2TyrProt/TyrProt, by GC–MS as well as by GC–tandem MS. GC–MS revealed a ratio of $2.44\pm2.34\times1{:}10^6$, whereas the ratio from GC–tandem MS analysis amounted to $1.48\pm0.65\times1{:}10^6$. The difference between these ratios was not statistically significant ($P=0.22$). The ratio of the values measured by GC–MS to those measured by GC–tandem MS was determined to be 1.56 ± 1.47.

3.5. Contribution of pronase to tyrosine, phenylalanine and 3-nitrotyrosine

The reproducibility of the enzymic proteolysis of total plasma proteins by pronase and the contribution of pronase to tyrosine, phenylalanine and 3-nitrotyrosine by self-proteolysis was investigated as follows. A total of 13 1-ml aliquots of a pooled plasma were ultrafiltered by centrifugation until ultrafiltrate volumes of $\sim780\pm60$ μl were obtained. The remaining protein fractions were diluted with 1-ml aliquots of a pronase solution in buffer B containing 6 mg of the enzyme. Sample volumes were added up to 2.5 ml with buffer B. Then 1-ml aliquots of these samples and two 1-ml aliquots of the pronase solution in buffer B (2.4 mg pronase per ml) were incubated at 37 °C for 20 h. All samples were ultrafiltered by centrifugation, 10-μl aliquots of the ultrafiltrates were diluted with 990-μl aliquots of the HPLC solvent, and protein-derived Tyr and Phe were determined by HPLC. The mean molar ratio of protein-derived Tyr to protein-derived Phe was determined to be 0.823 ± 0.056 (RSD 6.8%, $n=13$) for the plasma samples and 1.865 ± 0.025 (RSD 1.3%, $n=2$) for the pronase samples. Plasma protein-derived Tyr and Phe concentrations with regard to 1 ml plasma were determined as 5.95 and 7.23 m*M*, respectively. The concentration of albumin in plasma of healthy humans amounts to 440–660 μ*M* (i.e. 30–45 g/l). Quantitative proteolysis of albumin present in 1 ml of plasma would yield 7.9–11.9 m*M* Tyr and 13.6–20.5 m*M* Phe. Assuming that plasma albumin is the sole contributor to Tyr and Phe due to proteolysis, the extent of albumin proteolysis is calculated to be 50–75%. The theoretical molar ratio of TyrALB/PheALB of 0.58 disagrees with the measured mean ratio of 0.823 in the plasma samples.

Two 1-ml aliquots of a pronase solution in buffer B (6 mg pronase per ml) were incubated at 37 °C for 20 h. The concentrations of Tyr and Phe in the ultrafiltered digestion mixtures were determined to be 1.5 ± 0.02 and 0.98 ± 0.03 m*M*, respectively, yielding a mean Tyr/Phe molar ratio of 1.53. GC–tandem MS analysis for 3-nitrotyrosine in these samples yielded 3-nitrotyrosine at 21.9 ± 4.1 n*M*, i.e. with pronase contributing to 3-nitrotyrosine by 3.65 ± 0.68 pmol per mg protein. Since pronase apparently undergoes self-proteolysis in the absence of other proteins, it is likely that pronase has also contributed to the molar ratio of Tyr/Phe measured in the plasma samples as well as to plasma protein-derived 3-nitrotyrosine.

3.6. Estimation of basal plasma levels of 3-nitrotyrosinoalbumin

Considering a dilution factor of 5 for plasma and a recovery of ~50% for both affinity extraction [13] and enzymic proteolysis, the concentration of NO_2TyrALB in plasma of the 18 young volunteers involved in the study (Fig. 3) is estimated to be 23.6 ± 3.8 n*M* on day 1 and 24.7 ± 5.8 n*M* on day 15. Representative chromatograms from the GC–tandem MS analysis of NO_2TyrALB and NO_2Tyr in plasma of a healthy young volunteer are shown in Fig. 4. Within a retention time window of 6 min no other peaks appear in the chromatograms. The concentration of NO_2Tyr in this plasma sample was determined as 1.02 n*M*. The concentration of NO_2TyrALB in the hydrosylate was estimated as 1.8 n*M* which corresponds to a plasma concentration of

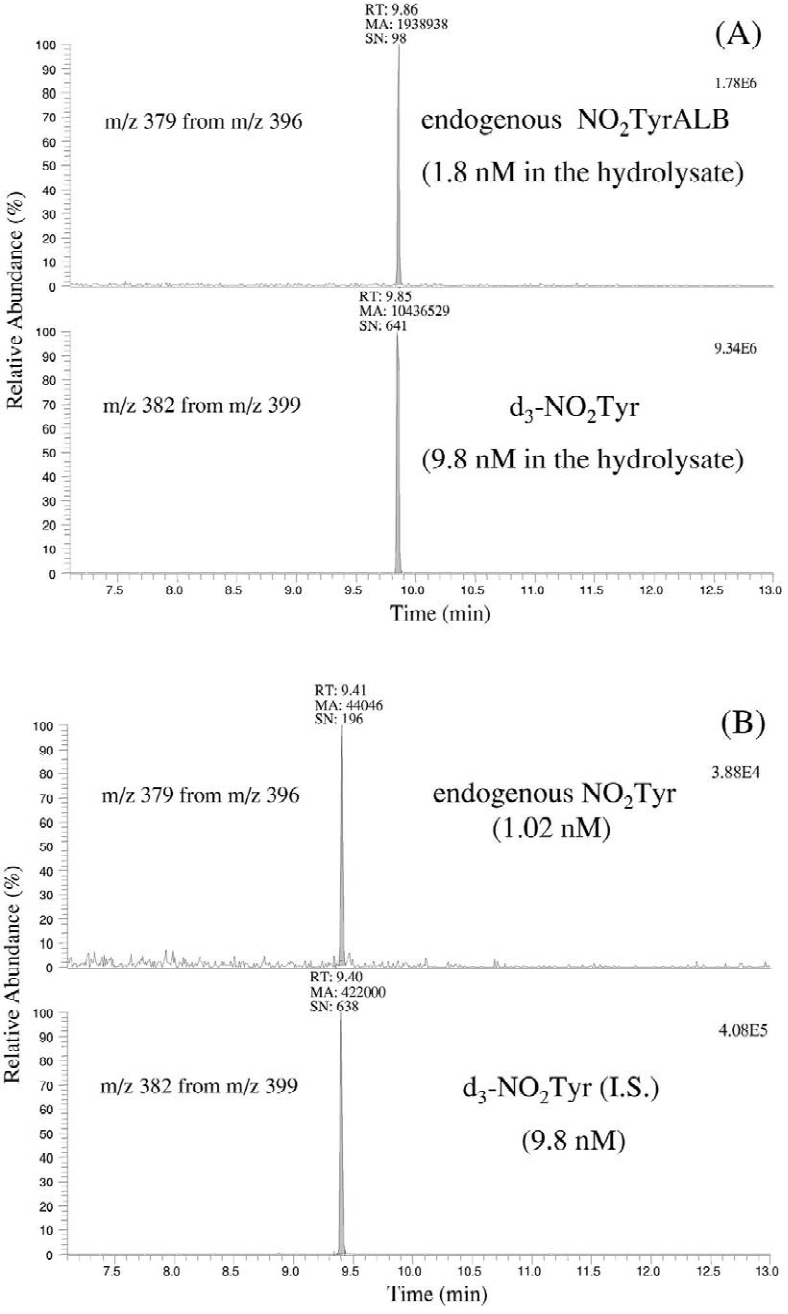

Fig. 4. Partial chromatograms from the GC–tandem MS analysis of a plasma sample from a healthy volunteer for NO_2TyrALB (A) and NO_2Tyr (B). Selected reaction monitoring of m/z 379 (from m/z 396) for endogenous NO_2Tyr and m/z 382 (from m/z 399) for d_3-NO_2Tyr of the *n*-propyl-*N*-pentafluoropropionyl-trimethylsilyl ether derivatives was performed. The difference in the retention time of the NO_2Tyr peaks in (A) and (B) resulted from the GC–tandem MS analysis on different days of the same year (i.e. 2nd August in B; 29th September in A).

36 n*M* (i.e. 1.8 n*M*×dilution factor×(extraction yield)$^{-1}$×(digestion yield)$^{-1}$, whereas the dilution factor is 5, i.e. 2 ml eluate:0.4 ml plasma, the recovery of ALB and NO_2TyrALB from affinity extraction is 50% or 0.5, and the yield of 3-nitrotyrosine from digested NO_2TyrALB is 50% or 0.5). This estimation is based on the assumption that only one tyrosine moiety of an albumin molecule is nitrated.

The identity of the GC peak corresponding in t_R to the derivative of synthetic 3-nitrotyrosine was elucidated by generation of a product ion mass spectrum by CAD of the parent ion $[M–TMSOH]^-$ at m/z 396 (Fig. 5). The product ion mass spectrum shows two product ions at m/z 379 and 261, and the undissociated parent ion at m/z 396, which were also observed from synthetic 3-nitrotyrosine [9]. This finding is in agreement with the results of Table 2 and indicates that simple GC–MS may suffice for quantification of endogenous NO_2TyrALB, unlike free NO_2Tyr [9].

4. Discussion

4.1. Methodological problems with the measurement of free and protein-associated 3-nitrotyrosine in human plasma

Since the recognition of 3-nitrotyrosine as a potential biomarker of nitrosative/nitrative oxidants in vivo [16] serious effort has been made to develop analytical methods for the quantification of free 3-nitrotyrosine (NO_2Tyr) as well as protein-associated 3-nitrotyrosine (NO_2TyrProt) in plasma of humans and animals. However, the analytical methods available to date yielded highly divergent values for circulating NO_2Tyr and NO_2TyrProt at the basal state, ranging from ~1 to 64 n*M* for NO_2Tyr and from 0 to 120 n*M* for NO_2TyrProt, independently of which analytical technology has been applied (Table 3). Similarly, highly diverging basal plasma levels have been reported for other *S*-nitros(yl)ated/nitrated proteins, notably *S*-nitrosoalbumin [17]. These discrepancies raise many questions [11,18]. Can these compounds actually be quantified accurately in human plasma? Is there any analytical method that allows for this? Do we measure facts or artifacts? Which values are valid? As long as these questions remain unanswered, i.e. no reference values for NO_2Tyr and NO_2TyrProt in human plasma have been established, the function of these compounds as reliable biomarkers for nitrosative/nitrative oxidative stress in vivo in humans cannot be tested. Consequently, the results and the impact of numerous clinical studies have to be called into question.

The wide range of basal plasma levels of NO_2Tyr and NO_2TyrProt, offered even by the use of MS-based analytical methods (Table 3) which are generally accepted to be characterized by inherent accuracy, is a crystal clear disclosure for the existence of various serious methodological problems in the quantification of circulating NO_2Tyr and NO_2TyrProt. These difficulties originate mainly from the physiological occurrence of NO_2Tyr and NO_2TyrProt at very low basal concentrations in human plasma. This forces indirect detection with the consequence of analytical methods becoming complex, time-consuming and costly. Presently there are numerous analytical methods available which, however, are producing highly diverging results. This makes clinical researchers uncertain and may influence them to take a decision in favor of the most simple, but not in favor of the most reliable, analytical method [11,18].

4.2. Artifactual formation of 3-nitrotyrosine and interferences

The potential artifactual formation of NO_2Tyr and NO_2TyrProt from acidification of ubiquitous nitrite and nitrate and Tyr- and TyrProt-containing plasma samples during sample preparation has been early recognized and reported [16]. The majority of investigators, in particular those applying MS-based techniques where sample derivatization is indispensable, have considered and excluded this source of artifacts in their analytical methods as much as possible [3,4,6,9,10,12]. Nevertheless, the wide range of basal plasma levels for NO_2Tyr (1– 64 n*M*), NO_2TyrProt (24–120 n*M*) and NO_2TyrProt/TyrProt (1–35×$1{:}10^6$) reported so far (Table 3) clearly indicates that the precautions taken to prevent artifactual formation from sample acidification have been insufficient and/or that there are additional sources of interferences.

In previous work we have convincingly demon-

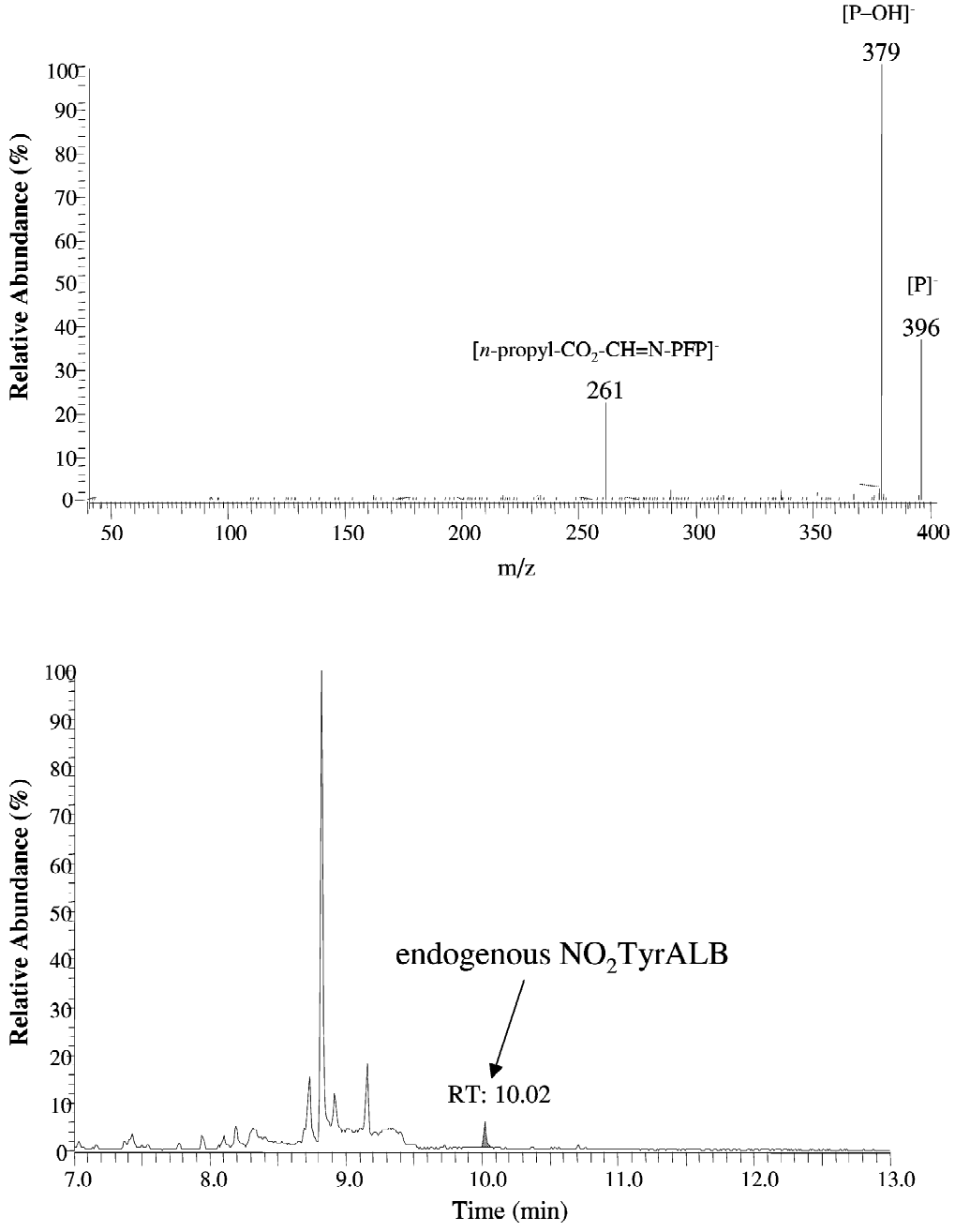

Fig. 5. GC–tandem MS mass spectrum (upper trace) of the *n*-propyl-PFP-TMS derivative, solid peak in the total ion current chromatogram (lower trace), eluting with the retention time of the *n*-propyl-PFP-TMS derivative of synthetic 3-nitrotyrosine (retention time, RT, 10.02 min). Human plasma (1 ml) was analysed for 3-nitrotyrosinoalbumin (NO_2TyrALB), proteins were digested by pronase, and the *n*-propyl-PFP-TMS derivatives were prepared and analysed by GC–tandem MS. The parent ion $[P]^-$ ($[M–TMSOH]^-$) at m/z 396 was subjected to CAD at a collision energy of 6 eV and the product ions were scanned between m/z 40 and 400. M, molecular mass of the *n*-propyl-PFP-TMS derivative of 3-nitrotyrosine (486 Da); PFP, pentafluoropropionyl; TMS, trimethylsilyl; TMSOH, trimethylsilanol.

Table 3
Summary of basal levels of NO_2Tyr and NO_2TyrProt and of the molar ratio of NO_2TyrProt/TyrProt in plasma of healthy humans and rats reported in the literature and cited in chronological order

NO_2Tyr (n*M*)	NO_2TyrProt (n*M*)	NO_2TyrProt/TyrProt ($\times 1{:}10^6$)	Species	Method	Reference
31	NM	NM	Human	HPLC–fluorescence	Kamisaki et al., 1996 [5]
NM	NM	0–1	Rat/Human	HPLC–ECD–UV	Shigenaga et al., 1997 [6]
ND	ND	NM	Human	ELISA	ter Steege et al., 1998 [7]
ND	120	NM	Human	ELISA	Khan et al., 1998 [8]
2.8	NM	NM	Human	GC–MS–MS	Schwedhelm et al., 1999 [9]
64	NM	35	Human	GC–MS	Frost et al., 2000 [4]
<4.4 (LOQ)	NM	NM	Human	LC–MS–MS	Yi et al., 2000 [3]
11	NM	NM	Human	GC–MS	Gaut et al., 2002 [10]
≤1.4 (LOQ)	NM	NM	Rat	LC–MS–MS	Delatour et al., 2002 [12]
0.7	24	1.55	Human	GC–MS–MS	Current study

NM, not measured; ND, not detected.

strated by GC–tandem MS that the *n*-propyl-PFP-TMS derivative of an unknown compound coelutes in GC with the *n*-propyl-PFP-TMS derivative of NO_2Tyr and has the same parent ion, i.e. m/z 396, which is monitored in GC–MS, but different product ions, strongly suggesting that GC–MS does not possess the necessary specificity to selectively measure plasma NO_2Tyr despite preceding HPLC analysis [9]. The present work confirms this finding for plasma NO_2Tyr and indicates that quantification of NO_2TyrALB in plasma by our GC–MS method may also be associated with similar interferences which, however, contribute to endogenous NO_2TyrALB much less than to NO_2Tyr (Tables 1 and 2; Fig. 5). The ratios of the concentrations measured by GC–MS to those measured by GC–tandem MS in the same plasma samples were (mean±SD) 2.87±3.14 ($n=6$) and 4.45±3.96 ($n=12$) for NO_2Tyr (Table 1), and 1.19±0.25 ($n=6$) for NO_2TyrALB/TyrALB (Table 2). Mean ratios clearly higher than 1 in combination with RSD values close to 100% for NO_2Tyr convincingly demonstrate that use of simple GC–MS in our method will reproducibly yield too high false values for NO_2Tyr (1- to 13-fold; Table 1). On the other hand, the mean ratio of 1.19 in combination with a RSD value of 21% suggests that NO_2TyrALB can be quantitated in human normal plasma by GC–MS in the present method with a higher accuracy than NO_2Tyr. It is, therefore, likely that similar interferences found in our method may also occur in other GC–MS methods and may result in highly overestimated levels, e.g. 64 n*M* [4] or 11 n*M* [10] versus 0.7–2.8 n*M* ([9] and the present study). Since, however, basal plasma values reported for NO_2Tyr and NO_2TyrProt by other groups are up to 30-fold higher than our own (Table 3), it must be concluded that the contributions of coeluting interfering compounds to these compounds in GC–MS are considerably higher than those found in our study. An explanation for the lower interference in our method could be the use of affinity column chromatography for selective extraction of NO_2TyrALB from plasma and HPLC for isolation of NO_2Tyr prior to GC–MS analysis.

We acknowledge that our method is complicated by the additional HPLC step for sample purification, and that high-throughput analysis is not possible with the present assay. However, expeditious analysis, as has been performed by other groups, has muddied our understanding of the importance of 3-nitrotyrosine as a marker of RNS. Therefore, choice of analytical quantitative methods should be directed to reliability rather than to simplicity and rapidity. As Tyr and NO_2Tyr exhibit similar chromatographic behavior, simple SPE might not suffice for efficient separation of very low amounts of NO_2Tyr from very large amounts of Tyr and other potential interfering compounds present in plasma under conditions, especially for pH, that avoid artificial formation of NO_2Tyr. We are convinced that HPLC is

currently required and is the most efficient method for the separation of NO_2Tyr from tyrosine, nitrate, nitrite, and presumably from other compounds that may interfere with the quantification of NO_2Tyr and NO_2TyrProt by GC–MS and/or GC–tandem MS. Despite use of GC–MS, Frost et al. [4] have measured the highest normal plasma levels so far reported for both NO_2TyrProt/TyrProt (i.e. $35\times 1{:}10^6$) and NO_2Tyr (i.e. 64 n*M*), the latter being even three times higher than those found by Kamisaki et al. [5] by means of HPLC (Table 3). Since the derivatives of both Tyr and NO_2Tyr were present in the same sample which was injected to quantify these compounds by GC–MS, it is possible that during the GC–MS analysis NO_2Tyr was artifactually formed or other compounds from plasma coeluted, interfered and contributed to endogenous NO_2Tyr.

4.3. Quantification of 3-nitrotyrosine by GC–tandem MS or GC–MS and LC–tandem MS

The work by Yi et al. [3] shows that their LC–tandem MS method detecting underivatized 3-nitrotyrosine does not apply to quantification of NO_2Tyr in human plasma at the basal state due to insufficient sensitivity (LOQ of 4.4 n*M*), indirectly supporting our levels of 0.7 n*M* (present study, $n=18$) to 2.8 n*M* ([9], $n=8$). LC–tandem MS analysis of NO_2Tyr as butyl ester has been shown by Delatour et al. [12] to increase sensitivity (LOQ of 1.4 n*M*). Unfortunately, this group has not determined NO_2Tyr in human normal plasma but only in rat plasma and found NO_2Tyr at 1.4–1.5 n*M*, which is the LOQ of the method, measurable in only four of eight rat plasma samples analysed. This finding is further strong support of the NO_2Tyr levels of 0.7–2.8 n*M* measured by us by means of a method involving HPLC and GC–tandem MS. Recently, LC–tandem MS analysis of strongly electron-capturing pentafluorobenzyl (PFB) derivatives of biomolecules including the amino acid phenylalanine has been shown to allow sensitive detection in the attomole range [19], like our GC–tandem MS for the *n*-propyl-PFP-TMS derivative of 3-nitrotyrosine [9]. It remains to be investigated whether LC–tandem MS analysis of 3-nitrotyrosine in human plasma as PFB derivative allows for its accurate quantification.

4.4. Enzymic and chemical hydrolysis of protein-associated 3-nitrotyrosine: estimation of concentrations of 3-nitrotyrosinated plasma proteins

Another fundamental difficulty in the measurement of nitrotyrosinated proteins in the circulation and in tissue is the unknown and most likely varying number of nitrated tyrosine molecules. The current generally accepted solution for this problem is the normalisation of protein-derived 3-nitrotyrosine (i.e. NO_2TyrProt) by the protein-derived tyrosine (i.e. TyrProt), i.e. the use of the molar ratio of NO_2TyrProt/TyrProt. Among the numerous circulating proteins we decided in favor of albumin because it is the most abundant and best investigated plasma transport protein. Measurement of NO_2TyrALB and TyrALB would, furthermore, facilitate a more reliable comparison of experimental data from various groups generated by using different analytical methods. Also, the availability of affinity column chromatography, e.g. HiTrapBlue Sepharose cartridges, offers the unique possibility to perform a single, highly selective extraction step for native and modified albumin molecules including NO_2TyrALB and *S*-nitrosoalbumin prior to individual quantitation.

For the generation of NO_2TyrALB and TyrALB we preferred enzymic proteolysis to chemical acidic or alkaline hydrolysis because the latter require drastic conditions for pH and temperature, e.g. heating in 6 *M* HCl at 110 °C for 24 h [16] or heating in 4 *M* NaOH at 120 °C for 16 h [4], conditions under which NO_2TyrALB may artifactually be formed or may degrade. Shigenaga et al. [6] have reported that the proteolytic enzyme pronase, which was also used by us in the present study, contributes to $<2\%$ of the tyrosine recovered, and that the rates of enzymic digestion for NO_2TyrProt and TyrProt are essentially the same. In our experiments, however, we found considerable contribution of pronase to tyrosine, phenylalanine and 3-nitrotyrosine by self-digestion in the absence of other proteins. We observed similar molar ratios for Tyr/Phe from enzymatically digested human plasma albumin after its affinity column extraction from plasma and from plasma proteins with molecular weights above 20 kDa which remained in the protein fraction after ultrafiltration, i.e. 0.72 (Fig. 3) and

0.82 (Section 3.5), respectively. This difference most likely results from the use of albumin and pronase at different amount ratios in the proteolytic mixtures of 6:1 and 5:1, respectively, with the pronase fraction being greater in the second experiment.

Assuming that both digested albumin and self-digested pronase contributed to the molar ratio of Tyr/Phe (R) measured in the proteolytic mixtures according to their mole fraction (N), Eq. (1) can be formulated:

$$N_{ALB} \times R_{ALB} + N_{PRO} \times R_{PRO} = R \quad (1)$$

where N_{ALB} and N_{PRO} are the mole fractions of albumin and pronase, respectively, in the proteolytic mixture, with $N_{ALB} + N_{PRO} = 1$, and R_{ALB} and R_{PRO} are the molar ratios of Tyr/Phe from digested albumin and pronase, respectively. Eq. (1) can be rearranged into Eq. (2):

$$R_{ALB} = (R - N_{PRO} \times R_{PRO})/N_{ALB} \quad (2)$$

Insertion of the values for R, R_{PRO}, N_{PRO} and N_{ALB} from the second experiment, i.e. $R = 0.823$, $R_{PRO} = 1.864$, $N_{PRO} = 0.167$ and $N_{ALB} = 0.833$, into Eq. (2) yields the value for R_{ALB} which amounts to 0.614. This R_{ALB} value is very close to the theoretical value of 0.58 and supports our assumption that albumin is the major contributor to Tyr and Phe in our method, but with pronase also contributing to Tyr and Phe by self-digestion. The deviation of the R_{ALB} value of 0.614 from the theoretical value of 0.58 by ~6% could have resulted from other digested plasma proteins and/or incomplete digestion of the native and nitrated albumin molecules.

The actual contribution of self-digested pronase in the presence of albumin and other plasma proteins to protein-derived 3-nitrotyrosine, tyrosine and phenylalanine is difficult to exactly determine. In the case of determining NO_2TyrALB, however, the contribution of pronase to these analytical parameters can be more precisely and accurately estimated than when measuring other plasma proteins in addition to NO_2TyrALB.

5. Conclusions

To date highly diverging values have been reported for NO_2TyrProt and NO_2Tyr in human plasma at the basal state. The most reliable data are provided by GC–tandem MS in combination with affinity column extraction of NO_2TyrALB from native plasma, enzymic digestion at neutral pH, and separation of protein-associated NO_2Tyr (from ultrafiltrate of digestion mixtures) and free NO_2Tyr (from plasma ultrafiltrate) by HPLC from Tyr and TyrProt, nitrite and nitrate, and numerous other potential interfering compounds. Despite this rigorous procedure, GC–MS is insufficiently specific for NO_2Tyr in human plasma at the basal state, but it is sufficiently specific for the quantification of NO_2TyrALB. Our GC–tandem MS-based methods yield unambiguous values for both NO_2Tyr and NO_2TyrALB which amount to 0.3–4.2 nM (range, $n = 29$) and 15–40 nM (range, $n = 18$), respectively, and for the molar ratio NO_2TyrALB/TyrALB which amounts to $0.5–3.5 \times 1{:}10^6$ (range, $n = 18$). These values are strongly supported by data generated by using LC–tandem MS-based methods from other groups. We recommend the use of these values as reference values for NO_2Tyr and NO_2TyrALB in normal human plasma.

Acknowledgements

E. Schwedhelm was the recipient of a graduate grant from Hannover Medical School. This study was supported in part by a grant from the Deutsche Forschungsgemeinschaft (grant Ts 60/2-1).

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