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Short communication

Sensitive and accurate analyses of free 3-nitrotyrosine in exhaled breath condensate by LC–MS/MS

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Abstract

The quantitative determination of 3-nitro-L-tyrosine, a biological marker for inflammatory processes, in exhaled breath condensate (EBC) is described. The clean-up and preconcentration was performed by solid phase extraction (SPE). After liquid chromatography the specific detection was performed by tandem mass spectrometry using electron spray ionisation and selected reaction monitoring (SRM). $^{13}C_9$-3-nitrotyrosine was used as an internal standard. For reliability, tests for the precision of the method, the losses during preparation, a test for nitrating artifacts and the comparibility of calibrants in EBC and buffer solution were performed. The calibration of the method was linear over a range of 10–500 pg/mL. The within-run coefficients of variation (CV) of the samples were found to be 8.4% at 25 pg/mL and 8.3% at 250 pg/mL. The day-to-day CV was found to be 11.2%. The limit of quantification was 3.9 pg/mL. The losses during preparation were 15%. The discrepancy between the calibration with EBC and buffer solution was below 10%. No artificial production of 3-nitrotyrosine was observed during the procedure. The application of the method on the EBC samples of healthy smokers ($N = 10$) and non-smokers ($N = 10$) showed no difference between the two groups. The concentration of 3-nitrotyrosine ranged between the limit of quantification and 184 pg/mL and was distinctly lower than data detected by an immunoassay procedure. The procedure was proven to be accurate, sensitive and in contrast to GC methods less elaborate and is recommended for the determination of 3-nitrotyrosine in exhaled breath condensate.

Keywords: Biological effect monitoring; Respiratory symptoms; Exhaled breath condensate; Oxidative stress; Nitric oxide

1. Introduction

Biological effect monitoring has become important in the early detection of diseases of the respiratory system [1,2]. For this purpose the use of exhaled breath condensate (EBC) has greatly increased in the last decade due to the non-invasive nature of its sampling. The exhaled breath condensate is collected by the cooling of expired air, which results in the condensation of expired water and low-volatile compounds. In the lung these substances are transferred into the air by nebulization and vaporisation. Thus, the investigation of exhaled breath condensate provides an approach for imaging the pathobiology of peripheral lung structures.

It is known that nitric oxide (NO) can react in vivo with the superoxide ion to produce peroxynitrite, a potent oxident with a half life of less than a second. The formation of peroxynitrite may be responsible for the cytotoxicity commonly attributed to nitric oxide. Peroxynitrite is considered by some authors to be the major reactive form of NO in vivo, especially under condition of inflammation where the production of oxygen free radicals is prominent [3]. Peroxynitrite, as well as myeloperoxidase generated nitrogen dioxide, reacts with free or protein bond tyrosine residues producing 3-nitro-L-tyrosine (3-NT), a stable end product (Fig. 1). In subjects with lung diseases free and protein-bonded nitrotyrosine was detected in serum, bronchoalveolar lavage and lung tissue [4–7]. First investigations on 3-NT in exhaled breath condensate were performed by the group of Kharitonov and co-workers for smoking and non-smoking healthy subjects [8], for patients with asthma [9] and patients with cystic fibrosis [10]. Using a specific enzyme immunoassay, they verified a mean 3-NT concentration of 6.3 ng/mL in exhaled breath condensates of healthy subjects and significant higher concentrations in patients with cystic fibrosis. However, the studies of Lärstad et al. [11] and Rothe et al. [12], which were

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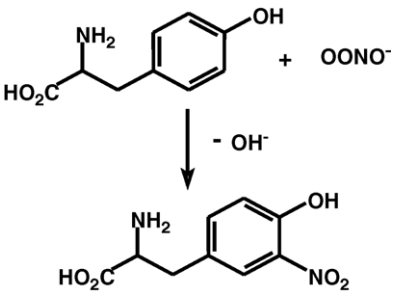

Fig. 1. Nitration of tyrosine by peroxynitrite.

performed with GC–tandem-MS and HPLC–MS techniques, suggested much lower concentrations of free nitrotyrosine in exhaled breath condensate.

Our goal was the development of a reliable and sensitive analytical procedure for the quantitative detection of free 3-NT in exhaled breath condensate by using high performance liquid chromatography and tandem mass spectrometry.

2. Experimental

2.1. Chemicals

3-Nitro-L-tyrosine was purchased from Cayman Chemical (Ann Arbor, MI, USA). L-Tyrosine was purchased in biochemical grade from Merck (Darmstadt, Germany). $^{13}C_9$-L-Tyrosine was purchased from Cambridge Isotope Laboratories (Andova, MA, USA). Ammonium acetate was purchased in Fractopur© grade from Merck (Darmstadt, Germany). All other substances and solvents were used in analytical grade. SPE cartridges of type C18 POLAR RP (100 and 500 mg) from Mallinckrodt Baker (Deventer, The Netherlands) were used for sample preconcentration and clean-up in the preparation of the internal standard, respectively.

The internal standard, $^{13}C_9$-3-nitrotyrosine, was synthesized according the preparation of Schwedhelm et al. [13]. $^{13}C_9$-L-Tyrosine (60 mmol) and sodium nitrate (180 mmol) were suspended in 600 μL of ice-cold water. Upon slow addition of 600 μL of sulphuric acid (97%) under stirring the colour of the cold solution changed to yellow. After one hour incubation in the ice-bath the pH of the solution was adjusted to 3 by the addition of 1700 μL aqueous solution of sodium hydroxide (5 M). The target substance was removed from the reaction solution by adsorption onto a C18 POLAR RP cartridge (500 mg) and elution by a solution of 2 mM NH_4Ac buffer and methanol (60/40, v/v) adjusted to pH 4.35. The purity of the reaction product in the eluent was proofed by LC–MS/MS and was found to be 99.7%.

2.2. Preconcentration and clean-up

For clean-up and preconcentration of the analytes solid phase extraction (SPE) was performed using reverse phase material. The C18 POLAR RP cartridges (100 mg) were preconditioned first by 4 mL of a solution of 2 mM NH_4Ac buffer (pH 4.35) and methanol (60/40, v/v) and second by 1 mL 2 mM NH_4Ac buffer (pH 4.35). Hundred microlitres internal standard solution ($^{13}C_9$-3-nitrotyrosine in 2 mM NH_4Ac, 2 ng/mL) and 50 μL 3 N acetic acid were added to 500 μL of exhaled breath condensate and the solution was transferred onto the cardridge. The cardridges were washed by 1 mL 2 mM NH_4Ac buffer (pH 4.35) and sucked to dryness. For elution 1.5 mL of a solution of 2 mM NH_4Ac buffer (pH 4.35) and methanol (60/40, v/v) were used. The eluate was concentrated to dryness by a stream of nitrogen and the residue was redissolved in 50 μL of a solution of 2.4 mM NH_4Ac buffer and methanol (85/15, v/v; adjusted at pH 3.4 with acetic acid). The solution was transferred into high recovery polypropylene vials (Agilent, Palo-Alto, CA, USA) for LC–MS/MS analysis.

Table 1
Selected reactions and conditions of tandem mass spectrometry

Analyte	SRM	DP (V)	EP (V)	CE (eV)
3-Nitrotyrosine (quantifier)	227 → 181	41	9	18
3-Nitrotyrosine (qualifier)	227 → 90	41	9	39
$^{13}C_9$-3-Nitrotyrosine	236 → 189	30	10	18

DP: declustering potential, EP: entrance potential, CE: collision energy.

2.3. HPLC conditions

Liquid chromatography was used for the separation of the analytes. The HPLC system consisted of a Agilent series 1100 (Agilent, Palo-Alto, CA, USA) equipped with a Zorbax Stable Bond Phenyl (100 mm × 2.1 mm, 3 μm) microbore column (Agilent, Palo-Alto, CA, USA) at a constant temperature of 30 °C. For the chromatography an isocratic eluent of 2.4 mM NH_4Ac buffer/methanol (85/15, v/v) adjusted at pH 3.4 with acetic acid was used with a flow-rate set at 220 μL/min. The injection volume was 20 μL.

2.4. Tandem MS conditions

The detection was performed by positive electrospray ionisation on a API 3000 tandem mass spectrometer (Applied Biosystems, Foster City, CA, USA). Spray voltage was set at 5 kV and the temperature of the auxiliary gas (dryer) at 500 °C. The nebulizer gas pressure was set to 10 psi. The acquisition was carried out in the selected reaction monitoring (SRM) mode and two characteristic transitions for nitrotyrosine and one for the internal standard were monitored. For the quantification of 3-nitrotyrosine, the reaction m/z 227 → 181 was selected. Additionally the reaction m/z 227 → 90 was used for qualifying purpose. For $^{13}C_9$-3-nitrotyrosine the reaction m/z 236 → 189 was monitored. The conditions for SRM are listed in Table 1. Resolution was set at 0.7 Da full width at half peak height with a 800 ms scan time per transition for reaction m/z 227 → 181 and 300 ms scan time per transition for the other reactions.

2.5. Preparation of standard curve

The 3-NT standard stock solution (1 mg/mL) was prepared by dissolving 10 mg of 3-nitro-L-tyrosine in 2 mM NH_4Ac buffer. The solution was further diluted with 2 mM NH_4Ac buffer in 100 ng/mL in order to be used as a working standard solution.

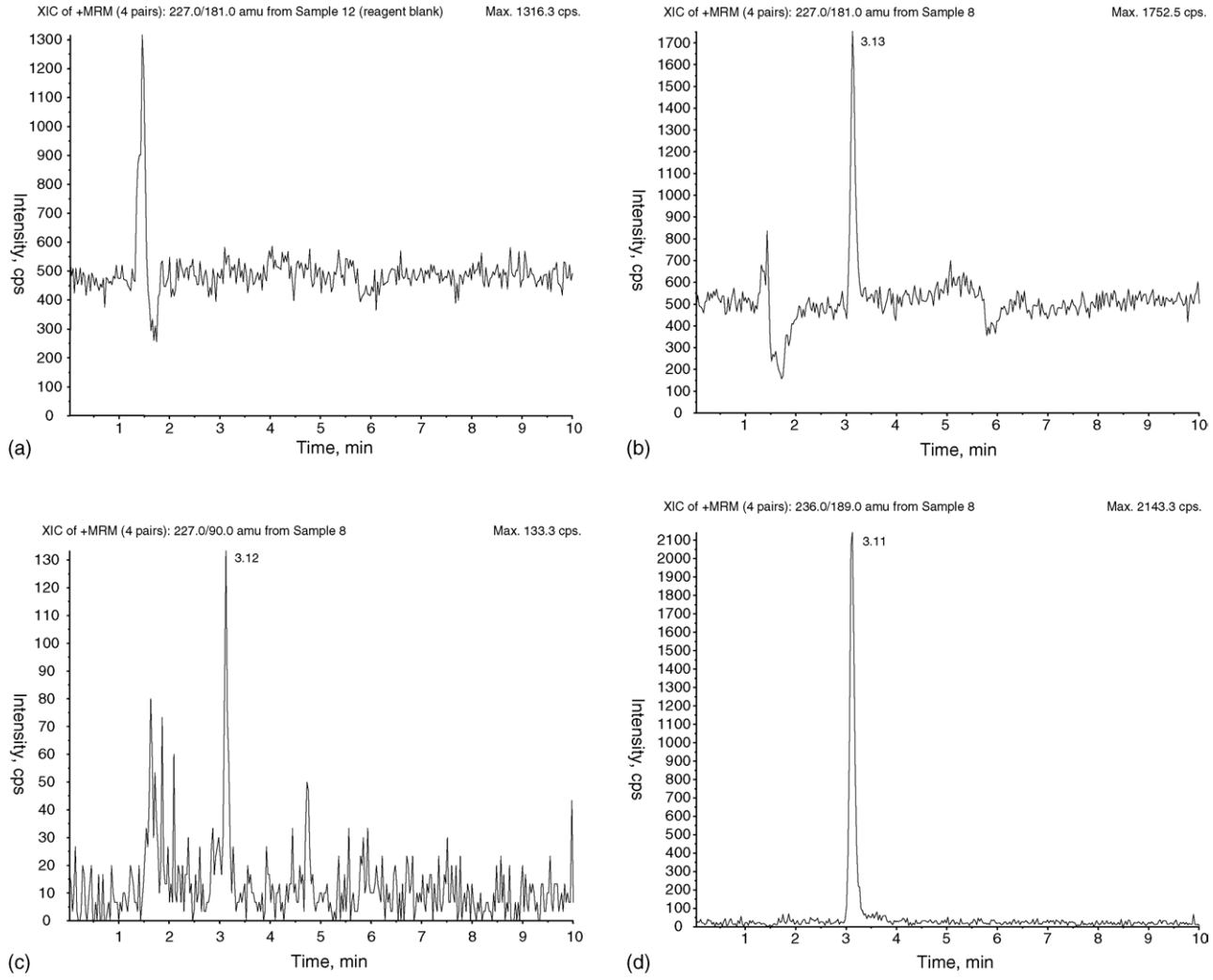

Fig. 2. Chromatograms of 3-nitrotyrosine quantifier reaction of a reagent blank (a) and of an EBC sample of 113 pg/mL (b); 3-nitrotyrosine qualifier reaction of the same EBC sample (c) and of $^{13}C_9$-3-nitrotyrosine reaction (d).

Calibrants with concentrations of 10, 25, 50, 75, 100, 150, 250 and 500 pg/mL were done by further dilution in 2 mM NH_4Ac buffer. The stock, working and calibration solution were stored at −20 °C and were proved to be stable under this condition over a period of 6 month.

2.6. Determination of precision and losses of the analyte

We determined the precision profile of the method by multiple analysis of spiked NH_4Ac solutions. The precision was analysed at both a concentration of 25 pg/mL and a concentration of 250 pg/mL. Ten samples of each concentration were analysed in parallel. The reproducibility was calculated as standard deviation and was expressed as coefficient of variation (CV). A pool of EBC samples were selected for the purpose of quality control. Each day one of these samples was analysed over a period of nineteen days and the results were used for the calculation of the coefficient of variation for day-to-day precision.

The recovery of the analyte after SPE preconcentration and clean-up was analysed by the comparison of calibration standards with and without SPE. For this six different calibration standards between 10 and 500 pg/mL nitrotyrosine in 2 mM NH_4Ac buffer underwent SPE in duplicate and were analysed by LC–MS/MS. For reference, six solutions of the analyte in a mixture of 2 mM NH_4Ac buffer (pH 4.35) and methanol (98/2, v/v; pH 3.4) were prepared at concentrations ten-fold higher than the standard solution with regard to the theoretical preconcentration factor. These solutions were injected directly into the LC–MS/MS system in triple.

2.7. Determination of the limit of quantification

We determined the detection limit and the limit of quantification according to ISO 11843 [14]. For the determination of the detection limit we analysed standard solutions of 10, 15, 25, 50 and 75 pg 3-NT/mL in 2 mM NH_4Ac buffer and the blank solution in triple. The calculation was performed on the basis of a linear calibration curve for SRM m/z 227 → 181. The limit of quantification was calculated from the detection limit accepting a relative measurement uncertainty of 33.3%.

2.8. Testing for nitration artifacts

To verify the accuracy of the method we tested a potential formation of 3-NT from tyrosine during the procedure. For this we applied our procedure to a solution of 50 μg tyrosine and 500 ng KNO_3 and 300 ng $NaNO_2$ in 500 μL 2 mM NH_4Ac buffer in triple and determined the probable formed 3-NT. For comparison the tyrosine solution was analysed in absence of nitrite and nitrate too.

2.9. Collection of exhaled breath condensate (EBC)

The procedure was applied to the EBC samples of 10 healthy non-smokers and 10 smokers, who had refrained from smoking two hours before EBC sampling (age: 20–40 years). Eleven individuals were investigated on two different days for the determination of intraindividual differences. Exhaled breath condensate was collected using the ECOSCREEN condenser from Jaeger (Hoechberg, Germany). During the sampling process (15 min) the tidal breathing was controlled by a specific spirometer (SpiroPro, Jaeger GmbH, Hoechberg, Germany) which was connected at the exhalation end of the condenser. Immediately after the collection of the EBC sample, the condensate was transferred into polypropylene vials and stored at −76 °C until analysis.

3. Results

Fig. 2 shows the chromatographic elution of 3-NT and $^{13}C_9$-3-nitrotyrosine in a reagent blank and in an EBC sample. In the reagent blank, 3-nitrotyrosine is absent as seen in Fig. 2a. Fig. 2b and c show the presence of endogenous 3-NT of about 113 pg/mL eluting at 3.1 min in a EBC sample for the SRM *m/z* 227 → 181 and *m/z* 227 → 90, respectively. Fig. 2d shows the chromatogram of the internal standard, which elutes at 3.1 min too.

In order to determine the absolute losses of nitrotyrosine during the solid phase extraction, the calibration curves with and without SPE were compared in Table 2. Without correction by the internal standard, the slope of the calibration curve with solid phase extraction was 85.0% of the slope for the calibration standards without SPE. After correction by the internal standard, the slope of the linear calibration curve was 98.8% the slope of the standards without SPE. Compared to the slope of the calibration curve of 3-NT standards in EBC the slope of the calibration in NH_4Ac buffer was 9.5% higher. Furthermore, the method was proven to be accurate when tested against artificial synthesis of nitrotyrosine under worst case conditions. In this experiment no additional nitrotyrosine was found.

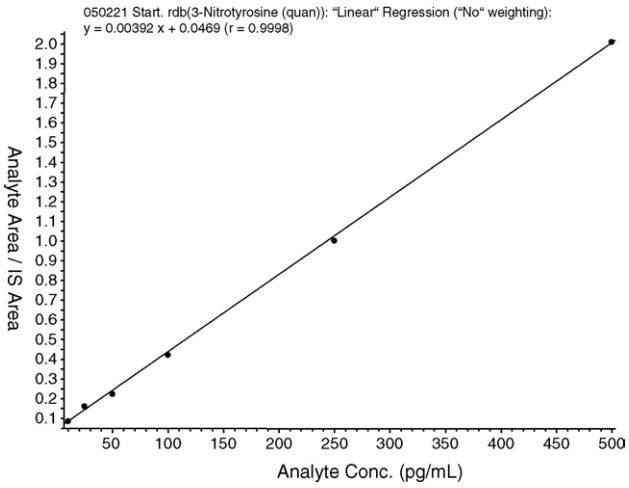

Fig. 3. Calibration curve for 3-nitrotyrosine.

Ratios of peak area of 3-NT/$^{13}C_9$-NT measured in the calibration solutions were linear over a range of 0–500 pg/mL (Fig. 3). The results of the analysis of replicate standard solution are shown in Table 3. The coefficients of variation for the within-run precision were found to be 8.4% at 25 pg/mL and 8.3% at 250 pg/mL. The coefficient of variation for the day-to-day precision was found to be 11.2% at a mean concentration of 68.4 pg/mL. On the basis of ISO 11843 the limit of quantification was determined to be 3.9 pg/mL for the described procedure and detection system.

Table 4 shows the 3-NT concentration found in the EBC of healthy subjects. In 94% of these EBC samples, nitrotyrosine was found to exceed the limit of quantification. No significant

Table 2
Comparison of calibration curves with and without SPE

	With SPE	Without SPE
Without correction by internal standard	$y = 76.564x + 18.545$	$y = 90.118x - 1865.6$
With correction by internal standard	$y = 0.00316x + 0.0119$	$y = 0.00320x - 0.0105$

Table 3
Results of the analysis of replicate standard solutions

Standard concentration (target value) (pg/mL)	Accuracy (relative deviation from target value) (%)	Precision (CV) (%)
10	+23.0	8.0
25	+3.4	9.0
50	−3.9	0.4
100	−3.0	0.1
250	+1.0	2.5
500	−0.3	2.0

Table 4
Concentration of 3-NT in EBC of healthy subjects (mean, minimum–maximum, in pg/mL)

	First exercise	Second exercise	Individual day-to-day difference
Smoker	57.0 ($n = 10$) <3.9–148.0	37.6 ($n = 5$) 10.6–63.3	48.1 2.7–74.6
Non-smoker	71.1 ($n = 10$) 11.7–172	69.1 ($n = 6$) <3.9–184.0	41.8 15.8–95.0

Table 5
Analytical procedures for the determination of 3-NT in human plasma

Biological material	Analytical technique	Detection limit	Mean levels in healthy subjects	Reference
Plasma	GC–MS	10 ng/ml	14 ng free NT/ml plasma	[15]
Plasma	GC–MS	18 pg/ml	2.4 ng free NT/ml plasma	[17]
Plasma	GC–MS/MS	6.8 pg/ml	0.16 ng free NT/ml plasma	[18]
Plasma	GC–MS/MS	28 pg/ml	0.16 ng free NT/ml plasma 5,4 ng NT in HSP[a]/ml plasma	[19]
Plasma	LC–FD	1.4 ng/ml	7 ng free NT/ml plasma	[20]
Plasma	LC–MS/MS	1 ng/ml	<1.0 free NT/ml plasma	[21]
Plasma/nasal lavage	Immunoassay	0.5 ng/ml	35 ng total NT[b]/ml plasma	[22]

[a] HSP: human serum protein.
[b] Sum of free NT and NT in HSP.

difference was found between the nitrotyrosine concentrations in EBC of smokers and non-smokers.

4. Discussion

The SB Phenyl column was selected as stationary phase for the chromatographic separation of the analyte because of its well-balanced retention abilities for aromatic substances. Thus, the elution of the analytes was performed by a low share of organic solvent and without gradient. As seen in the chromatogram, the signals of 3-NT and internal standards are free of interference. Furthermore, the isocratic conditions of the mobile phase resulted in a short total run-time which favoured this method for the application in practice.

According to the accuracy tests, 15% of analyte were lost during the solid phase extraction. However, the procedure includes two mechanisms for compensation. First, an isotope labelled internal standard was used whose physical properties are very similar to the properties of the native substance. This compensation effect was demonstrated by the equal calibration curves of the quotient of nitrotyrosine and internal standard for calibration standards with and without SPE (Table 2). Second, external standards were analysed in parallel with the samples whereby the calibration curve was also affected by the same recovery rate like the samples. The calibration was performed by using NT buffer solution because of the low disposability of the biological matrix. However, the agreement with the results of spiked EBC demonstrates a high reliability of this kind of calibration. Moreover, the assurance of the method was proved against artificial synthesis of nitrotyrosine. Because tyrosine could partly be nitrated under strong acid conditions and in the presence of nitrite but not under conditions of pH 3–5 [15], extreme acid conditions were not used in our method. Using a worst-case simulated solution of tyrosine, nitrite and nitrate no additional amounts of nitrotyrosine were formed during the procedure. As seen in the chromatogram the qualifier mass reaction of 3-nitrotyrosine could be used for concentration of about of 100 pg/mL and higher, which provides a favoured possibility for the verification of increased NT levels.

Since nitrotyrosine was assumed to be a biological marker for inflammatory processes, several methods for the quantification of free and protein-bonded 3-nitrotyrosine in biological materials have been developed [16]. Most applications were performed for the analysis of nitrotyrosine in plasma. The studies published in the last decade regarding this application, indicate an increase of sensitivity and specificity of the methods. With regard to the limit of detection (LOD), the highest sensitivity was reached by using tandem mass spectrometry (Table 5). Furthermore, an increase of specificity was demonstrated by the lowering of the mean nitrotyrosine level in plasma. Delatour and co-workers published a LC–MS/MS method for the simultaneous determination of 3-nitrotyrosine and tyrosine in plasma proteins of rats with a detection limit of 316 pg/mL plasma for 3-nitrotyrosine [23]. However, their procedure has the disadvantage of a dilution of the sample by the factor of 1.2. In the method of Yi et al. [21] plasma samples were also not concentrated before LC–MS/MS analysis. For this specimen the detection limit of NT was 1 ng/mL (Table 5). In contrast, the sensitivity of our method was supported by a ten-fold concentration of the sample by the combination of solid phase extraction and evaporation. Moreover, the low complexity of the EBC specimen favoured the low limit of detection (LOD) which is comparable with the LOD of the GC–MS/MS methods for plasma analysis. The advantage of our method to the GC–MS/MS methods is the short route of preparation of the samples, whereas nitrotyrosine has to be derivatized for gaschromatographic analysis.

We found NT values in exhaled breath condensate of healthy subjects which were distinctly lower than in the literature. However, with the exception of the abstracts mentioned [11,12], data relating to the NT concentration in EBC have been, until this time, published by just one group [8–10]. Using a commercial immunochemical analytical technique, they have found a mean NT concentration in the EBC of healthy subjects of 6.3 ng/mL. The authors stated a detection limit of 3.9 and 1.3 ng/mL after preconcentration. According to the manufacture of the immunoassay used in these studies the test system did not recognize nitrated tyrosine bound in proteins. In a recent comparison of the application of an ELISA and a GC/MS method on the determination of 8-isoprostane in urine it was found that the immunoassay yielded much higher values of 8-isoprostane than the GC/MS assay and small correlation was found between their results [24]. Moreover, the comparison of total nitrotyrosine in plasma analysed by GC–MS/MS [19] and immunoassay technique [22] show distinctly higher values for the immunoassay as well. Furthermore, it should be emphasized that the concentration of free nitrotyrosine in plasma was below 1 ng/mL with respect to the analysis by structural techniques (Table 5). Because EBC represents a highly diluted matrix, similar or lower

concentration should be expected for EBC. On the other hand our NT levels are in agreement with the NT concentration presented by Lärstadt and co-workers in their presentation [11]. They mentioned a mean value of 149 ± 93 pg/mL for four healthy, non-smoking volunteers, which corresponds with the range of our results.

In conclusion, the new procedure on the basis of clean-up and preconcentration by SPE, liquid chromatography and tandem mass spectrometry was proved to be reliable and sensitive for the determination of free 3-nitrotyrosine in exhaled breath condensate. The results for healthy subjects demonstrate the necessity of a highly sensitive method for the determination of nitrotyrosine in EBC. Moreover, these results confirm the problems of the immunochemical technique to generate accurate results, as mentioned by other authors [11,16–17]. Due to the proven reliability the new LC–MS/MS method, this is the method of choice for the accurate quantification of 3-nitrotyrosine in EBC. Due to individual day-to-day variance of the parameters, an improved standardization of sampling conditions is recommended if 3-nitrotyrosine should be used for biological monitoring of inflammatory reactions in the airways.

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