ELSEVIER

Journal of Chromatography B, 661 (1994) 185–191

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Quantification of nitrite and nitrate in human urine and plasma as pentafluorobenzyl derivatives by gas chromatography–mass spectrometry using their ^{15}N-labelled analogs

D. Tsikas*, R.H. Böger, S.M. Bode-Böger, F.-M. Gutzki, J.C. Frölich

Institute of Clinical Pharmacology, Hannover Medical School, Konstanty-Gutschow-Strasse 8, D-30623 Hannover, Germany

First received 17 May 1994; revised manuscript received 16 August 1994

Abstract

For the quantification of nitrite and nitrate, the stable metabolites of L-arginine-derived nitric oxide (NO) in human urine and plasma, we developed a gas chromatographic–mass spectrometric (GC–MS) method in which [^{15}N]nitrite and [^{15}N]nitrate were used as internal standards. Endogenous nitrite and [^{15}N]nitrite added to acetone-treated plasma and urine samples were converted into their pentafluorobenzyl (PFB) derivatives using PFB bromide as the alkylating agent. For the analysis of endogenous nitrate and [^{15}N]nitrate they were reduced to nitrite and [^{15}N]nitrite, respectively, by cadmium in acidified plasma and urine samples prior to PFB alkylation. Reaction products were extracted with toluene and 1-μl aliquots were analyzed by selected-ion monitoring at m/z 46 for endogenous nitrite (nitrate) and m/z 47 for [^{15}N]nitrite ([^{15}N]nitrate). The intra- and inter-assay relative standard deviations for the determination of nitrite and nitrate in urine and plasma were below 3.8%. The detection limit of the method was 22 fmol of nitrite. Healthy subjects ($n = 12$) excreted into urine 0.49 ± 0.25 of nitrite and 109.5 ± 61.7 of nitrate (mean ± S.D., μmol/mmol creatinine) with a mean 24-h output of 5.7 μmol for nitrite and 1226 μmol for nitrate. The concentrations of nitrite and nitrate in the plasma of these volunteers were determined to be (mean ± S.D., μmol/l) 3.6 ± 0.8 and 68 ± 17, respectively.

1. Introduction

Over the last few years nitrite and nitrate have become of great interest because they have been shown to be the stable metabolites of the highly unstable endothelium derived relaxing factor (EDRF) nitric oxide (NO) [1,2]. The origin of NO has been demonstrated to be the amino acid L-arginine [3]. NO formation is associated with an activation of the soluble guanylate cyclase and release of cGMP [4–6] leading to vascular smooth muscle relaxation [7]. Recently, urinary and plasma nitrite/nitrate as well as cGMP have been shown to be suitable index parameters to assess L-arginine-derived NO formation and to monitor its effects on the vascular system in vivo [8].

There exist numerous analytical methods to measure nitrite/nitrate in aqueous solutions but only a few of them are applicable to biological

* Corresponding author.

0378-4347/94/$07.00
SSDI 0378-4347(94)00374-2

fluids. Among them are also gas chromatographic–mass spectrometric (GC–MS) methods which utilize [^{15}N]nitrite and [^{15}N]nitrate as internal standards and which are mainly based on the nitration of aromatic compounds [9,10]. However, the application of these methods to nitrite/nitrate determination in biological fluids, especially in plasma, is associated with many interferences [11]. In the present study, we describe a rapid, interference-free and highly sensitive GC–MS method for quantitative determination of nitrite and nitrate in urine and plasma samples of humans. The method is based on the conversion of nitrite into α-nitro-pentafluorotoluene using pentafluorobenzyl (PFB) bromide as the alkylating agent and ^{15}N-labelled nitrite as the internal standard. The same derivatization procedure was used for nitrate and ^{15}N-labelled nitrate following their reduction by cadmium to nitrite and ^{15}N-labelled nitrite, respectively. Urinary 24-h excretion of nitrite and nitrate as well as their levels in plasma were determined by this method in healthy volunteers.

2. Experimental

2.1. Chemicals and reagents

α-Bromo-2,3,4,5,6-pentafluorotoluene (PFB-Br) was obtained from Aldrich (Steinheim, Germany). The sodium salts of [^{15}N]nitrite and [^{15}N]nitrate (each 99 atom% ^{15}N) were obtained from MSD Isotopes Merck Frosst Canada (Montreal, Canada). Toluene of analytical-reagent grade was purchased from Merck (Darmstadt, Germany). Acetone, the sodium salts of nitrite and nitrate (Riedel-de Haen, Seelze, Germany) were of analytical-reagent grade. Double-distilled water was used for the preparation of aqueous solutions of nitrite and nitrate and of the internal standards.

2.2. Volunteers and sample collection

Nitrite and nitrate were measured in urine and plasma samples obtained from venous blood of healthy female volunteers receiving a standardized (group A) and uncontrolled nitrate diet (group B). In group A (mean age 25.9 ± 1.0 years, $n = 10$), urine samples were obtained from spontaneous micturition on two different days within an interval of eight days. One day before sample collection the volunteers received a standardized nitrate diet with an intake of 26 mg nitrate per day. In group B (mean age 24.8 ± 0.8 years, $n = 12$), urine was collected for 24 h and stored at 4°C during the collection period. Blood was immediately centrifuged at 4°C (5000 *g*, 10 min) and plasma stored on ice. Two 1-ml aliquots each from urine and plasma samples were separately spiked with the internal standards, frozen immediately and stored at −20° until analysis. GC–MS analysis showed that under these conditions no loss of nitrite from blood was observed within the first 2 h of storage. However, when blood was incubated immediately at 37°C endogenous nitrite was oxidized quantitatively to nitrate with a half-life of approx. 12 min. Nitrate was found to be stable in urine and plasma samples when they were incubated for 2 h at 37°C or stored for 24 h at 4°C.

2.3. Derivatization procedure

Nitrite and ^{15}N-labelled nitrite were converted to their PFB derivatives by modifications of previously described methods [12,13]. Briefly, 100-μl aliquots of urine or plasma spiked with the corresponding internal standards [^{15}N]nitrite and [^{15}N]nitrate (250 ng for urine and 25 ng for plasma samples) were treated with 400 μl of acetone cooled to −20°C. Following immediate centrifugation (3000 *g*, 5 min) and decantation the supernatant was treated with 5 μl of PFB-Br and the mixture was allowed to react for 60 min at 50°C. After cooling to room temperature acetone was removed under nitrogen and reactants were extracted into 1 ml of toluene from which 1-μl aliquots were injected onto the GC–MS system. For the determination of nitrate, to a 100-μl aliquot of a urine or plasma sample spiked with the internal standard 5 mg of cadmium were added and subsequently acidified by addition of 20 μl of a 20 vol.% acetic acid

solution. Reduction of nitrate to nitrite was performed after reaction at room temperature for 15 min by gently mixing with a mechanical shaker. The supernatant of the centrifuged sample (3000 *g*, 5 min) was decanted and derivatized as described above for nitrite without neutralization.

2.4. GC–MS conditions

GC–MS analyses were performed on a gas chromatograph 9611 interfaced with a triple-stage quadrupole mass spectrometer (TSQ 45) of Finnigan MAT (San Jose, CA, USA) operating in the single-stage quadrupole mode under negative-ion chemical ionization (NICI) conditions with methane as reagent gas (65 Pa). Helium was used as carrier gas at a pressure of 50 kPa. The temperature of the fused-silica capillary column (OV1701, 25 m × 0.36 mm I.D., film thickness 0.25 μm; Macherey-Nagel, Düren, Germany) was held at 60°C for 2 min and then increased to 100°C at a rate of 5°/min and to 250°C at a rate of 30°/min. Both PFB-NO_2 and PFB-$^{15}NO_2$ eluted from the column at the same time. Injector, interface and ion-source were held at 280°C, 280°C and 130°C, respectively. The ionization energy was 90 eV and the electron current 200 μA. The electron multiplier voltage was set at 1.2 kV.

3. Results

3.1. GC–MS analysis

In the mass spectrum of synthetic PFB-NO_2 (Fig. 1) the most abundant ion was NO_2^- at *m/z* 46. Other less intense signals were observed at *m/z* 226 ($[M-H]^-$) and *m/z* 181 ($[PFB]^-$). Except for *m/z* 181 the signals observed in the mass spectrum of PFB-$^{15}NO_2$ were increased by 1 *m/z*. The ions at *m/z* 46 for nitrite (nitrate) and *m/z* 47 for [^{15}N]nitrite ([^{15}N]nitrate) were used for quantitation in the selected-ion monitoring mode. Partial GC–MS chromatograms from the analysis of nitrite plus nitrate in urine of a healthy volunteer are shown in Fig. 2.

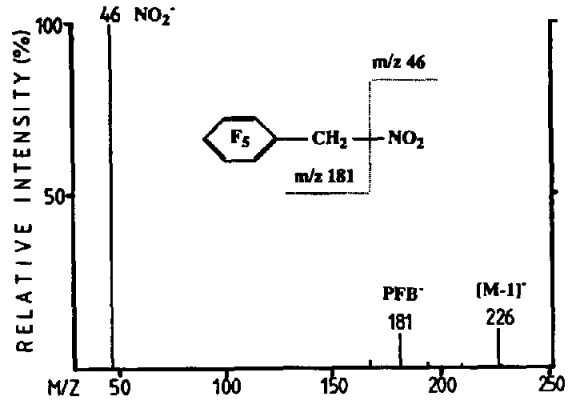

Fig. 1. Negative-ion chemical ionization mass spectrum of synthetic α-nitro-pentafluorotoluene.

GC–MS chromatograms from the analysis of nitrite and nitrite plus nitrate in plasma of a healthy volunteer are shown in Fig. 3. These figures show that no other compounds interfere with the determination of nitrite and nitrate in urine and plasma samples.

3.2. Precision

The precision of the overall procedure was determined by analyzing five (unspiked) samples of plasma and urine for nitrite and nitrate from the same sample pool. The mean concentrations

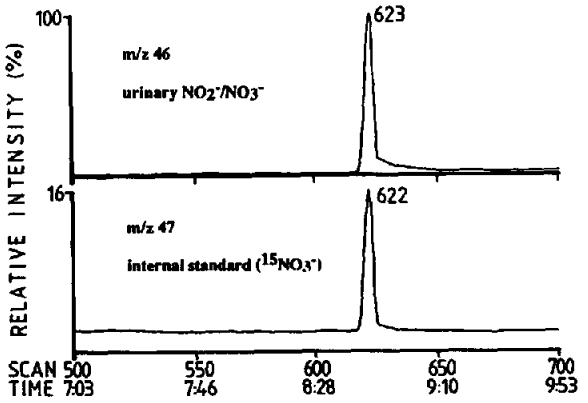

Fig. 2. A typical partial GC–MS chromatogram from the analysis of nitrite plus nitrate in the urine of a healthy volunteer as PFB derivatives. The concentration of the internal standard ($^{15}NO_3^-$) in the urine sample (100 μl) was 2.5 mg/l (39.7 μmol/l).

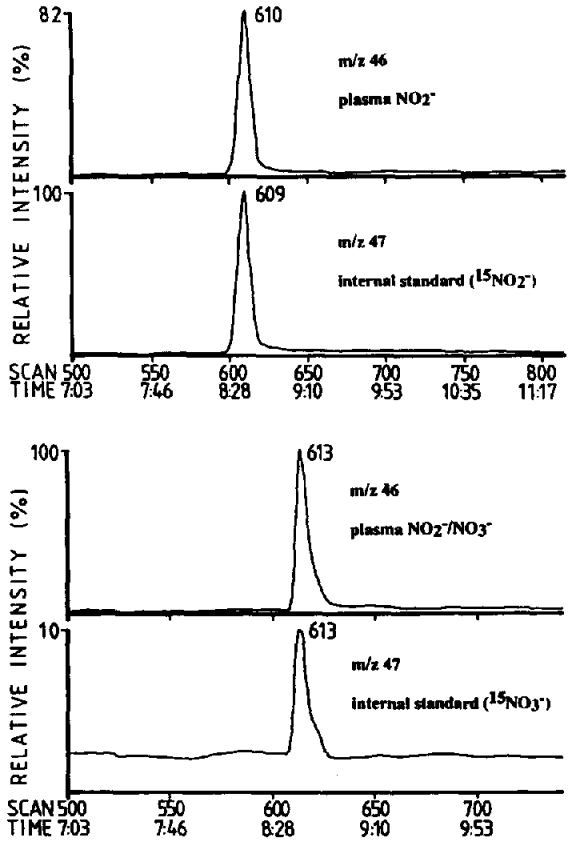

Fig. 3. Typical partial GC–MS chromatograms from the analyses of nitrite (upper panel) and nitrite plus nitrate (lower panel) in plasma samples (each 100 μl) of a healthy volunteer as PFB derivatives. The concentrations of $^{15}NO_2^-$ and $^{15}NO_3^-$ in the plasma samples were 0.25 mg/l (5.32 μmol/l) and 0.25 mg/l (3.97 μmol/l), respectively.

of nitrite and nitrate in the plasma of a volunteer were determined to be 3.4 and 50.8 μmol/l with an R.S.D. of 3.5% and 2.3%, respectively. In the same volunteer the mean concentrations of nitrite and nitrate in the urine were determined to be 8.9 and 750 μmol/l with an R.S.D. of 3.8% and 1.8%, respectively. When nitrite and nitrate were analyzed in five 100-μl aliquots of twice-distilled water their mean concentrations were determined to be 1.2 and 2.8 μmol/l with an R.S.D. of variation of 2.1% and 1.9%, respectively. These values representing the nitrite and nitrate content of the reagents used were substracted from the values obtained in biological samples. The R.S.D. from sixfold injection of 5 pg of synthetic PFB-NO_2 (22 fmol nitrite) was 0.8%. This amount was detectable at a signal-to-noise ratio of 50:1.

3.3. Accuracy

Linearity was checked by spiking 25 ng of $^{15}NO_2^-$ or 250 ng of $^{15}NO_3^-$ to each 0.1-ml aliquot of a sample (aqueous solution, urine, plasma) to which was added separately up to 200 ng nitrite/0.1 ml and up to 10 μg nitrate/0.1 ml. For all matrices linearity was observed between the ratio m/z 46 to m/z 47 and the amount added within the range investigated with coefficients of correlation better than 0.996.

The accuracy of the method was assessed by analyzing in triplicate seven 100-μl aliquots of a urine sample each spiked with 250 ng/0.1 ml of [^{15}N]nitrate and with up to 26 μg/0.1 ml of nitrate. The data given in Table 1 and the very good correlation between the concentration of nitrate measured (y) and the concentration of nitrate added (x) ($y = 31.683 + 1.018x$, $r^2 = 0.999$), indicate the high accuracy of the method and the quantitative relative recovery for nitrate. The y-axis intercept is due to endogenous nitrate (31.7 mg/l). Similar results were also obtained for plasma samples and aqueous buffered solutions of nitrate.

Table 1
Accuracy of the method for the determination of nitrate in human urine

Nitrate added (mg/l)	Nitrate found (mean ± S.D., $n = 3$) (mg/l)	R.S.D. (%)
0	32.4 ± 0.98	3.04
50	82.7 ± 1.59	1.92
80	111.5 ± 3.30	2.96
110	145.9 ± 3.14	2.15
150	182.7 ± 4.21	2.31
190	224.7 ± 4.17	1.85
260	297.5 ± 4.69	1.58
Mean ± S.D.		2.26 ± 0.56

Table 2
Concentrations of nitrite and nitrate in plasma of twelve healthy volunteers with uncontrolled nitrate diet (group B)

Volunteer	Nitrite (μmol/l)	Nitrate (μmol/l)
1	4.5	57.1
2	4.6	75.6
3	2.4	60.3
4	3.2	62.5
5	3.1	62.6
6	3.5	47.8
7	2.8	114.7
8	4.2	79.5
9	3.6	72.3
10	5.3	65.7
11	3.2	67.1
12	3.4	50.8
Mean ± S.D.	3.65 ± 0.84	68.0 ± 17.4

3.4 Concentrations of nitrite and nitrate in plasma and urine of humans

The concentrations of nitrite and nitrate in plasma samples of twelve healthy volunteers with uncontrolled nitrate diet (group B) and the 24-h excretion of nitrite and nitrate in these volunteers are summarized in Tables 2 and 3, respectively. The concentrations on two different days of nitrite and nitrate in plasma and of nitrite plus nitrate in urine samples of ten healthy volunteers with standardized nitrate diet (group A) are listed in Table 4. In urine and plasma of volunteers on standardized low-nitrate diet significantly ($p < 0.02$; unpaired t-test; Table 4) lower nitrite and nitrate concentrations were measured compared with those determined in the volunteers with uncontrolled nitrate diet. This may result from the different intake of nitrite/nitrate present in food by the volunteers.

4. Discussion

Sensitive determination of nitrite in aqueous solutions as PFB derivative by GC with electron-capture detection has been demonstrated [12]. However, this method does not allow–except when additional purification steps are used [13]–determination of nitrite and nitrate in biological

Table 3
Excretion of nitrite and nitrate in the urine of healthy volunteers with uncontrolled nitrate diet (group B)

Volunteer	Nitrite		Nitrate	
	μmol/24 h	μmol/mmol creatinine	μmol/24 h	μmol/mmol creatinine
1	10.5	0.73	1422	98.2
2	2.2	0.42	858	163.8
3	8.2	0.71	1458	127.3
4	4.3	0.33	1351	105.0
5	2.5	0.17	953	68.1
6	9.9	1.02	2648	271.2
7	6.0	0.16	1185	31.1
8	6.6	0.61	1361	124.4
9	4.9	0.32	997	65.7
10	3.8	0.52	782	106.0
11	4.5	0.48	787	84.3
12	5.6	0.42	915	69.1
Mean ± S.D.	5.7 ± 2.7	0.49 ± 0.25	1226 ± 513	109.5 ± 61.7

24-h Output (μmol/24 h) and concentrations of nitrite and nitrate relative to creatinine (μmol nitrite or nitrate/mmol creatinine).

Table 4
Concentrations of nitrite and nitrate in plasma and urine and relative to creatinine concentration of nitrite + nitrate in urine of ten healthy volunteers on two different days receiving standardized nitrate diet (group A)

Volunteer	Plasma				Urine	
	Nitrite (μmol/l)		Nitrate (μmol/l)		Nitrite + nitrate (μmol/mmol creatinine)	
	Day A	Day B	Day A	Day B	Day A	Day B
1	1.7	2.6	34.5	68.2	18.3	20.0
2	1.1	1.9	20.6	29.5	58.8	95.7
3	2.4	3.3	43.8	20.3	45.8	25.7
4	1.5	1.5	30.5	34.0	48.4	42.5
5	2.2	1.3	36.8	21.6	94.7	58.9
6	1.5	1.7	33.2	32.6	55.9	80.2
7	2.4	2.6	63.2	30.2	77.3	34.2
8	1.3	1.3	38.2	57.7	45.6	59.8
9	2.2	1.9	41.9	21.1	29.6	109.0
10	1.7	1.5	38.2	33.1	19.7	41.5
Mean ± S.D.	1.8 ± 0.4	1.9 ± 0.6	38.1 ± 10.9	34.8 ± 15.9	49.4 ± 24.1	56.7 ± 29.9
p-Value[a]			0.0001	0.0002	0.009	0.0228

[a] p-Values were calculated by comparing the data of this table with those of the Tables 2 and 3 applying the unpaired t-test.

fluids due to interferences by coeluting substances from urine or plasma. The present study demonstrates that GC–MS is an excellent analytical tool for the highly sensitive determination of nitrite and nitrate in urine and plasma samples of humans as their PFB derivatives at a high degree of accuracy. Nearly complete fragmentation of the strongly electron capturing PFB-NO_2 (Fig. 1) and the use of stable isotope-labelled internal standards enable nitrite and nitrate to be easily and sensitively quantitated in complex biological matrices such as urine and plasma (Figs. 2 and 3). With regard to other GC–MS methods based on the nitration of aromatic compounds such as benzene and 1,3,6-trimethoxybenzene [11,14] the present method has the major advantage to allow accurate determination of nitrite in the presence of high excess of nitrate. Because the nitrite levels both in plasma and in urine are much lower than the nitrate levels and since these levels are within the range of the blank the determination of nitrite is extremely difficult and highly inaccurate by these methods (own unpublished results).

Our data on the concentration of nitrite and nitrate in human urine and plasma lie in the same range as reported by several groups [8,14,15]. The urinary nitrite and nitrate excretion rates with respect to creatinine measured by us lie in the same range as the values reported by Green et al. [14] and more recently by Kanno et al. [8] and Hibbs et al. [16] applying the method based on the Griess reaction. The plasma values for nitrite and nitrate obtained by our method are comparable with those reported by these and other groups [17,18]. On the other hand, there exist a number of studies in which nitrite was either not detectable in plasma or its concentration was clearly below 1 μmol/l [18–20]. Such discrepancies, especially regarding nitrite plasma levels, may be due to different intake of nitrite/nitrate by the volunteers involved in each study and also due to differences in sample generation, storage and treatment.

Intravenous infusion of L-arginine in healthy persons has been shown to result in a decrease in blood pressure most probably due to a NO-specific mechanism [8,21]. In these studies the decrease in blood pressure was found to correlate more tightly with the increase in urinary

excretion of nitrate than of cGMP. Thus, this finding and the difficulty in measuring authentic NO in plasma suggest that nitrite and nitrate, the stable metabolites of NO, are more suitable index parameters for NO formation from L-arginine. The observation that after infusion of L-arginine nitrite plus nitrate excretion into urine increased but plasma nitrite or nitrate did not, together with the relative short half-life of nitrite in blood (see Ref. [22] and this study) suggest that the most appropriate matrix for assessment of NO formation in man is urine. Our results show that the concentrations of nitrite and nitrate both in urine and plasma seem to depend upon the intake of nitrite and nitrate by foods. This observation and the finding that intravenous infusion of L-arginine (30 g) in humans led to only a 1.6- to 1.8-fold increase in urinary nitrite and nitrate excretion [8,21] suggest that clinical studies on the role of EDRF/NO in health and disease in which nitrite and/or nitrate function as index-parameter for EDRF/NO formation must be carefully performed under standardized low nitrite/nitrate diet. Also, in the detection methods chemical and reagents of lowest nitrite/nitrate content should be used.

5. Conclusions

From all evidence available at this time urinary nitrite/nitrate appears to be a reliable parameter to assess non-invasively whole body nitric oxide (NO) synthesis in man. The GC–MS technique described in this paper allows highly accurate quantitation of nitrite and nitrate in urine and plasma and should be useful to monitor effects of diseases and drugs on NO synthesis in man.

Acknowledgement

The technical assistance of Miss A. Otten is gratefully acknowledged.

References

[1] R.M.J. Palmer, A.G. Ferrige and S. Moncada, *Nature*, 327 (1987) 524.
[2] L.J. Ignarro, G.M. Buga, K.S. Wood, R.E. Byrus and G. Chandhuri, *Proc. Nat. Acad. Sci. USA*, 84 (1987) 9205.
[3] H.H.H.W. Schmidt, H. Nau, W. Wittfoht, J. Gerlach, K.E. Prescher, M.M. Klein and E. Böhme, *Eur. J. Pharmacol.*, 154 (1988) 213.
[4] W.P. Arnold, C.K. Mittal, S. Katsuki and F. Murad, *Proc. Nat. Acad. Sci. USA*, 1977 (1975) 3203.
[5] C.A. Gruetter, B.K. Barry, D.S. McNamara, D.Y. Gruetter, P.J. Kadowitz and L.J. Ignarro, *J. Cycl. Nucleot. Res.*, 5 (1979) 211.
[6] E. Böhme, G. Grossmann, J. Herz, A. Mülsch, C. Spies and G. Schulz, in P. Greengard, G.A. Robinson, R. Paoletti and S. Nicosia (Editors), *Advances in Cyclic Nucleotides and Protein Phosphorylation Research*, Raven Press, New York, NY, 1984, p. 259.
[7] R.F. Furchgott, *Annu. Rev. Pharmacol. Toxicol.*, 24 (1984) 175.
[8] K. Kanno, Y. Hirata, T. Emori, K. Ohta, S. Eguchi, T. Imai and F. Marumo, *Clin. Exp. Pharmacol. Physiol.*, 19 (1992) 619.
[9] R. Iyengar, D.J. Stuehr and M. Maretta, *Proc. Nat. Acad. Sci. USA*, 84 (1987) 6369.
[10] F.-M. Gutzki, D. Tsikas, U. Alheid and J.C. Frölich, *Biol. Mass Spectrom.*, 21 (1992) 97.
[11] J.W. Tesch, W.R. Rehg and R.E. Sievers, *J. Chromatogr.*, 126 (1976) 743.
[12] H.-L. Wu, H.-S. Chen, S.-J. Lin, W.-R. Hwang, K. Funazo, M. Tanaka and T. Shono, *J. Chromatogr.*, 269 (1983) 183.
[13] D. Tsikas and J.C. Frölich, *Fresenius J. Anal. Chem.*, 344 (1992) 256.
[14] L.C. Green, D.A. Wagner, J. Glogowski, P.L. Skipper, J.S. Wishnok and S.R. Tannenbaum, *Anal. Biochem.*, 126 (1982) 131.
[15] M. Rath and J.C. Kranz, Jr., *J. Pharmacol. Exp. Ther.*, 76 (1942) 27.
[16] J.B. Hibbs, Jr., C. Westenfelder, R. Taintor, Z. Vavrin, C. Kablitz, R.L. Baranowski, J.H. Ward, R.L. Menlove, M.P. McMurry, J.P. Kushner and W.E. Samlowski, *J. Clin. Invest.*, 89 (1992) 867.
[17] M. Zuber and R. Miesel, *Innere Medizin*, 48 (1993) 244.
[18] Å. Wennmalm, G. Benthin and A.-S. Petersson, *Br. J. Pharmacol.*, 106 (1992) 507.
[19] Å. Wennmalm, B. Lanne and A.-S. Petersson, *Anal. Biochem.*, 187 (1990) 359.
[20] A.J. Farrell, D.R. Blake, R.M.J. Palmer and S. Moncada, *Annals Rheum. Dis.*, 51 (1992) 1219.
[21] S.M. Bode-Böger, R.H. Böger, A. Creutzig, D. Tsikas, F.-M. Gutzki, K. Alexander and J.C. Frölich, *Clin. Sci.*, 87 (1994) 303.
[22] N.J. Parks, K.A. Krohn, C.A. Mathis, J.H. Chasko, K.R. Geiger, M.E. Gregor and N.F. Reck, *Science*, 212 (1981) 58.