ELSEVIER

Journal of Chromatography B, 715 (1998) 441–444

JOURNAL OF
CHROMATOGRAPHY B

Discussion

Measurement of nitrite and nitrate in plasma, serum and urine of humans by high-performance liquid chromatography, the Griess assay, chemiluminescence and gas chromatography–mass spectrometry: Interferences by biogenic amines and N^G-nitro-L-arginine analogs

Dimitrios Tsikas*, Ilka Fuchs, Frank-Mathias Gutzki, Jürgen C. Frölich

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

Received 24 April 1998; accepted 29 May 1998

Abstract

In this paper, the HPLC method for the measurement of nitrite and nitrate in serum of humans newly reported by El Menyawi et al. is discussed, especially in regard to the extremely low nitrate levels measured in serum of healthy humans. From the discussion, it is concluded that: (1) Biogenic amines at physiological concentrations do not significantly interfere with the batch Griess assay. (2) The HPLC method of El Menyawi et al. does not reveal accurate levels for serum nitrate. (3) In serum and plasma of healthy humans, nitrate ranges within 15–70 μ*M*. (4) Exogenous N^G-nitro-L-arginine analogs can interfere with the measurement of nitrate in human plasma and urine by the batch Griess assay, chemiluminescence and GC–MS; interferences can be effectively eliminated by solid-phase extraction on cation-exchangers. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Griess assay; Nitrite; Nitrate; N^G-nitro-L-arginine analogues; Biogenic amines; Amines

1. Introduction

Nitrate and nitrite, the oxidative products of nitric oxide (NO), physiologically occur in plasma and urine [1]. Measurement of nitrate and nitrite in these matrices, especially of nitrate in urine [2], is a reliable approach to assess NO formation in vivo [1]. The most frequently used methods for nitrate and nitrite include the spectrophotometric detection based on the Griess reaction, chemiluminescence and gas chromatography–mass spectrometry (GC–MS) (reviewed in [1]). Alternative methods for measurement of nitrate and nitrate in plasma or serum of humans include high-performance liquid chromatography (HPLC) ([1,3–5], and references therein) and capillary electrophoresis [6]. Recently, a further analytical method based on HPLC has been reported by El Menyawi et al. [7]. By means of this method, the sum of nitrite and nitrate concentration in serum of healthy volunteers has been determined as only 1.1 ± 0.05 μmol l^{-1} [7]. To our knowledge, this is the lowest reported level for the sum of nitrite and nitrate in serum of healthy humans from the use of assays dependent and independent on the Griess

*Corresponding author.

0378-4347/98/$19.00 © 1998 Elsevier Science B.V. All rights reserved.
PII: S0378-4347(98)00326-0

reaction [1]. The extremely low serum nitrate levels in healthy humans measured by El Menyawi et al. have been explained as a result of the elimination by solid-phase extraction (SPE) and HPLC of physiological biogenic amines that may cause increased nitrite and nitrate levels when measured by the Griess assay. It has been shown previously that measurement of nitrite and nitrate in human plasma or serum by assays related to the Griess reaction may be associated with many problems [1]. In a direct comparison of a GC–MS method with a batch Griess assay, similar to that used by El Menyawi et al. in their work [7], we found that the batch Griess assay gives lower and not higher levels for nitrate in both human plasma and human urine than the GC–MS method [1]. We, therefore, would like to present data to clarify these discrepancies.

2. Results and discussion

El Menyawi et al. [7] have reported that various physiological biogenic amines both in aqueous solution and in serum interfered with the Griess assay when performed as described by Schmidt et al. [8]. Compared to the results obtained by their HPLC method, El Menyawi et al. [7] have found both in aqueous solution and in plasma of malaria patients that the presence of biogenic amines gave rise to 100- to 1000-fold higher concentrations of nitrite when measured by the batch Griess assay. El Menyawi et al. have reported in their work that the Griess reagent reacts with free biogenic amines to form derivatives that show identical visible light spectra, making thus impossible differentiation between nitrite and biogenic amines. Unfortunately, no data have been given for the particular compounds and their concentration used in the experiments of this group. In consideration of the low concentration of free biogenic amines in human plasma and the very high concentration of the Griess reagents, it seems to be unlikely that physiological biogenic amines effectively react with nitrite and the reagents used in the Griess assay to form highly absorbing chromophors. Furthermore, it seems to be highly unlikely that the reaction products of the Griess reagents with biogenic amines absorb 100–1000 times stronger light around 540 nm than the corresponding reaction product of nitrite. Indeed, utilizing a similar batch Griess assay to that used by El Menyawi et al., we found that serotonin, one of the most abundant biogenic amines in human plasma occurring at concentrations of about 1 μM, decreased nitrite levels in aqueous solutions (range 0 to 100 μM) by 3% at 10 μM and by 12.5% at 100 μM of serotonin. The decrease could result from the reaction of nitrite with serotonin which competes with the Griess reagents, e.g. with sulfanilic acid. These results are in complete contradiction to those of El Menyawi et al. [7]. Serotonin at 100 μM was found not to interfere in the GC–MS method that uses PFB bromide (data not shown).

In consideration of the similar molar absorption coefficients of nitrate and nitrite at 214 nm [4], the HPLC chromatograms shown in figure 1 of the work of El Menyawi et al. which were obtained from the analysis of a serum sample of a patient suggests that nitrite is present in serum at substantially higher concentrations than nitrate. This fully contradicts the data reported for nitrite and nitrate in serum of patients. Moreover, the HPLC chromatograms in their figure 1 suggest that the recovery of nitrate from serum after the two SPE extraction procedures is substantially lower than the reported 90% [7]. Under the assumption that the concentration of nitrate in serum of the malaria patients were within the range of 12–18 μM, it is highly questionable that this HPLC method allows measurement of serum nitrate below this range. Measurement of nitrate in biological fluids by the Griess assay and other methods including GC–MS requires reduction of nitrate to nitrite, e.g. by cadmium [1]. We agree with El Menyawi et al. that the reduction step in the Griess and other assays except in GC–MS methods, where a stable isotope-labeled internal standard is used, is very crucial and can lead to inaccurate, and as a rule, lower nitrate levels in human plasma and urine [1]. Nevertheless, there exist a large number of publications in which plasma nitrate levels of the order of 15–70 μM have been reported as measured by the Griess assay (reviewed in [1]). This range has been confirmed by GC–MS previously [1,3,9]. During the present work was performed, we analysed by GC–MS [1] plasma of fourteen healthy humans for nitrate and measured mean levels of 35.9 μM (range 24.2–51.2) at an accuracy of 99% and an impreci-

sion of 1.5%. Furthermore, Wennmalm et al. have measured nitrate in plasma of healthy humans of the order of 44 μ*M* by HPLC and UV detection at 214 nm [3]. Leone et al. have measured similar serum nitrate levels by capillary electrophoresis [6]. It is notable that in both of these techniques, nitrate was measured without any derivatization. By contrast, El Menyawi et al. have reported that the sum of nitrite and nitrate in serum of their healthy volunteers was only 1.1±0.05 μ*M* [7]. Even in the malaria patients, for which elevated nitrate serum levels of the order of 40 μ*M* and above were measured by Nussler et al. [10] and Cot et al. [11], El Menyawi et al. have reported nitrate concentrations in the range of only 12–18 μ*M* [7]. It seems, therefore, to be highly unlikely that the nitrate levels measured by El Menyawi, in particular those in serum of healthy humans, are correct.

Greenberg and co-workers have reported that nitro containing L-arginine analogs such as the inhibitors of the NO synthase N^G-nitro-L-arginine methyl ester (L-NAME) and N^G-nitro-L-arginine (L-NNA) interfere both with the Griess assay and the chemiluminescence assay of nitrite and nitrate in plasma and urine [12]. We found that both L-NAME and L-NNA also interfered in the GC–MS measurement of nitrate in human urine when derivatization of nitrate was performed by using concentrated sulfuric acid and trimethoxybenzene [13] (Table 1). In the GC–MS method that uses derivatization of nitrite by pentafluorobenzyl bromide [9], no interference at all by L-NAME and L-NNA (each 1 m*M*) was observed for nitrite in human plasma (data not shown), indicating that the N^G-nitro group of these compounds does not decompose to nitrite under the derivatization conditions. On the other hand, L-NAME and L-NNA were found to positively interfere with the measurement of nitrate by this GC–MS method in human urine and plasma (Table 1). Since L-NAME and L-NNA do not physiologically occur in human plasma and urine, these compounds would not interfere under normal conditions. In the case of using these compounds in vitro or in vivo, removal of these compounds from the matrices is indispensable, and can effectively be achieved by SPE (Table 1).

In summary, physiological serum concentrations of biogenic amines such as serotonin do not interfere considerably with the measurement of nitrate in plasma by the batch Griess assay. The too low nitrate levels measured in serum of healthy and diseased humans by Menyawi et al. suggest that the HPLC method of this group is not accurate. L-NAME and L-NNA interfere with the measurement of nitrite and nitrate by the batch Griess assay, chemiluminescence and GC–MS. Elimination of these compounds prior to derivatization is absolutely necessary and can easily be achieved by SPE on cation-exchangers.

Table 1
Effect of L-NAME and L-NNA on the measurement of nitrate in human urine and human plasma by GC–MS analysis of the pentafluorobenzyl derivatives [1] or of the trimethoxynitrobenzene [13] with and without preceding SPE on 3-ml aromatic sulfonic acid Bakerbond cartridges (J.T. Baker, The Netherlands)

Matrix/Treatment	Nitrate concentration (μmol l^{-1}), mean±S.D., $n=4$	
	without SPE	with SPE
Urine (unspiked)	672±59	653±48
Urine+L-NAME (4.6 m*M*)	1061±82	628±81
Urine+L-NNA (4.6 m*M*)	1122±101	664±58
Urine (unspiked)	796±65	816±73
Urine+L-NAME (1 m*M*)	1083±95	784±120
Urine+L-NNA (1 m*M*)	995±110	815±67
Plasma (unspiked)	29.6±10.5	31.6±12.5
Plasma+L-NAME (1 m*M*)	184±25	32.8±10.3
Urine+L-NNA (1 m*M*)	173±18	30.5±12.8

The concentrations of L-NAME and L-NNA were each 4.6 m*M* for method [13] and each 1 m*M* for method [1].

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft (grant TS 60/2-1).

References

[1] D. Tsikas, F.-M. Gutzki, S. Rossa, H. Bauer, Ch. Neumann, K. Dockendorff, J. Sandmann, J.C. Frölich, Anal. Biochem. 244 (1997) 208.
[2] R.H. Böger, S.M. Bode-Böger, U. Gerecke, F.-M. Gutzki, D. Tsikas, J.C. Frölich, Clin. Exp. Pharmacol. Physiol. 23 (1996) 11.
[3] Å. Wennmalm, G. Benthin, A.-S. Petersson, Br. J. Pharmacol. 106 (1992) 507.
[4] S.A. Everett, M.F. Dennis, G.M. Tozer, V.E. Prise, P. Wardmann, M.R.L. Stratford, J. Chromatogr. A. 706 (1995) 437.
[5] M.N. Muscará, G. de Nucci, J. Chromatogr. B. 686 (1996) 157.
[6] A.M. Leone, P.L. Francis, P. Rhodes, S. Moncada, Biochem. Biophys. Res. Commun. 200 (1994) 951.
[7] I. El Menyawi, S. Looareesuwan, S. Knapp, F. Thalhammer, B. Stoiser, H. Burgmann, J. Chromatogr. B. 706 (1998) 347.
[8] H.H.H.W. Schmidt, T.D. Warner, M. Nakane, U. Förstermann, F. Murad, Mol. Pharmacol. 41 (1992) 615.
[9] D. Tsikas, R.H. Böger, S.M. Bode-Böger, F.-M. Gutzki, J.C. Frölich, J. Chromatogr. B. 661 (1994) 185.
[10] A.K. Nussler, W. Eling, P.G. Kremsner, J. Inf. Dis. 169 (1994) 1418.
[11] S. Cot, P. Ringwald, B. Mulder, P. Miailhes, J. Yap-Yap, A.K. Nussler, W.M.C. Eling, J. Inf. Dis. 169 (1994) 1417.
[12] S.S. Greenberg, J. Xie, J.J. Spitzer, J. Wang, J. Lancaster, M.B. Grisham, D.R. Powers, T.D. Giles, Life Sci. 57 (1995) 1949.
[13] F.-M. Gutzki, D. Tsikas, U. Adelheid, J.C. Frölich, Biol. Mass Spectrom. 21 (1992) 97.