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Discussion

Reply to ‘‘Measure of nitrite and nitrate in plasma serum and urine of humans by HPLC, the Griess assay, chemiluminescence and GC–MS’’, by Tsikas et al.

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Sir,

We appreciate that our recently published paper concerning serum nitrite/nitrate measurements by a newly developed HPLC-method [1] could attract the attention of our German colleagues and was a stimulus for reevaluation. As some of the conclusions drawn by this group in this issue of the *Journal of Chromatography B* are not in concordance with our point of view we would like to comment on some of the remarks made by Tsikas et al. to elucidate the situation.

In our opinion the basis for controversies was the low serum nitrate levels reported in our paper. We certainly agree with the authors that these are one of the lowest concentrations detected and documented, yet. But we would like to recall that serum nitrite and nitrate levels in general could vary over a broad range of magnitude and are considerably influenced by a large variety of parameters including diet, inhalation of NO, urea cycle metabolism, gut metabolism, endogenous reduction etc. [2–4]. Even differences due to environmental pollution are reported [5]. The actual outcome of serum nitrite and nitrate measurements therefore is extremely dependant on the collective of probands chosen for investigation. The recent study was performed in a Thai community. To our knowledge there are no data available which contradict our results regarding a similar experimental setting. These low concentrations by HPLC were initially suspicious, but spiked samples with known amounts of nitrite or nitrate lead to recovery rates between 87% and 92%. Therefore, excess losses due to the purification process seemed to be unlikely. One explanation could be that the serum nitrite and nitrate concentrations reported in the literature were too high, since the data were generated with the Griess-assay, which is known to be error prone.

We proposed a substantial higher nitrite concentration indicated by the Griess assay when biogenic amines were present in the sample which is contradicted by Tiskas et al. The Griess-assay does not result in higher nitrite levels due to interactions between biogenic amines and nitrite. The crucial reaction is mediated via a metabolite, the diazonium cation, which subsequently reacts with the coupling component of the Griess-assay [6–8]. Whether this diazonium cation originates from nitrite or from biogenic amines or from other sources is of no

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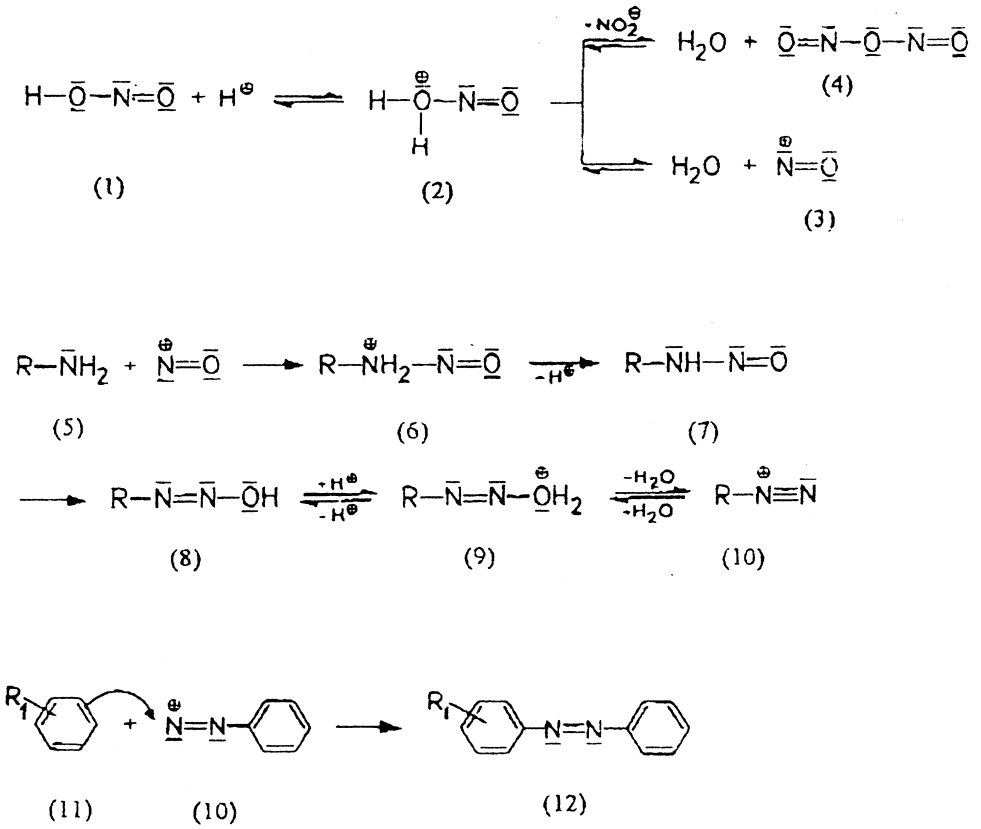

Fig. 1. Reaction pathway for the generation of the diazonium cation and diazo coupling.

relevance as the coupling component does not discriminate by source. Furthermore we do not implicate that the resulting structures form chromophors, which show substantially altered absorbing properties. The increase of absorbance is based upon a cumulative effect, where the absorbance by nitrite and the absorbance derived from biogenic amines is summarised (see for example Figs. 2–4). To clarify the situation we would like to recall the chemical pathway leading to the diazonium cation and the underlying reactions in the Griess assay. The schema shown in Fig. 1 describes the nitrosation of a wide range of aromatic primary and secondary amines. Compounds 2, 3 and 4 may be involved in an attack on nucleophilic centres present in amines (compound 5) or aminoderivatives leading via a series of reactions to a diazonium cation (compound 10). Aromatic amines and amides and even α-aminoacids present in biological systems may undergo azo coupling under the conditions suitable for the Griess assay [9].

The intention of the left graph of our paper solely

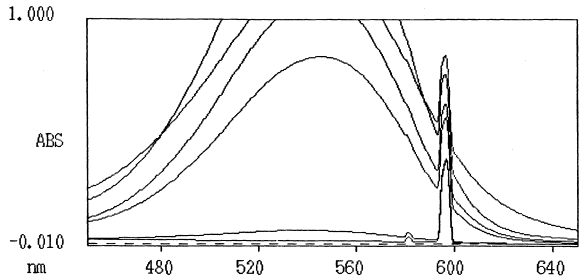

Fig. 2. Griess-assay in water: Absorption spectra of a solution containing 10 μM nitrite and different catecholamine metabolites. From bottom to top: 1. H_2O; 2. 10 μM NO_2^-; 3. 10 μM NO_2^- +10 μM 4-OH-3-MeO-mandelic acid; 4. 10 μM NO_2^- +10 μM 4-OH-3-MeO-phenylacetic acid; 10 μM NO_2^-; 5. 10 μM NO_2^- +10 μM 3-OH-4MeO-mandelic acid; 6. 10 μM NO_2^- +10 μM 5-OH-indol-3-acetic acid.

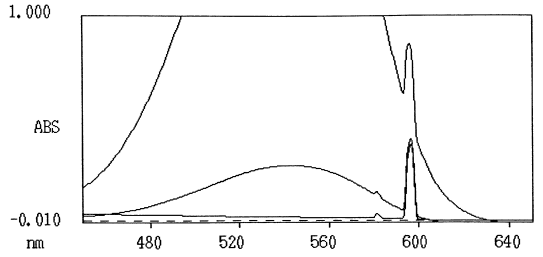

Fig. 3. Griess-assay in serum: Absorption spectra of a solution containing 10 μM nitrite and catecholamine metabolite. From bottom to top: 1. serum; 2. 10 μM NO_2^-; 3. 10 μM NO_2^-+10 μM 4-OH-3-MeO-mandelic acid.

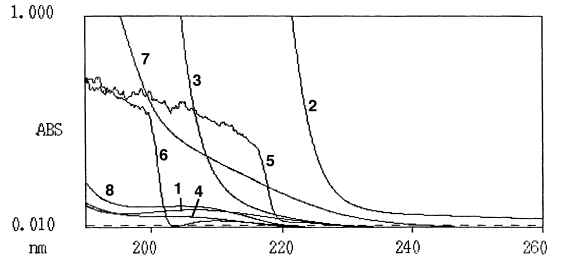

Fig. 5. Absorption spectras of nitrite and nitrate in different solutions. 1) 5 μM NO_2^- in H_2O, 2) 5 μM NO_2^- in KOH, 3) 5 μM NO_2^- in KCl, 4) 5 μM NO_3^- in H_2O, 5) 5 μM NO_3^- in KOH, 6) 5 μM NO_3^- in KCl, 7) 5 μM NO_2^- in methane sulfonic acid/KOH, 8) 5 μM NO_3^- in methane sulfonic acid/KOH.

was to visualize the result of the HPLC applying untreated serum samples onto the ionexchange column. This drawing should show the urgent need for purification of the sample for obtaining valid data. The peaks visible cannot be attributed to a distinct constituent of the sample. Nitrite and nitrate show different absorbance patterns with different maximas according to the applied buffering system (see Fig. 5). Indeed, both compounds show a similar extinction coefficient in water, but they have different absorption patterns in the sulfonic acid buffer we used in our experiments. The absorption maximum for nitrate is at 199 nm and for nitrite at 214 nm in our system. Thus, measurements at the maximum absorbance wavelength for the first constituent will automatically reduce the resulting HPLC peak of the other one, assuming an equal concentration of both components. Interpretation of the data is facilitated by comparison with peak patterns of a standard concentration of the compound under investigation, which we did not show. Therefore a higher ''nitrite'' peak in comparison to a smaller ''nitrate'' peak does not inevitably mean you achieve higher nitrite concentrations in the serum sample. This conclusion would be valid only after calculation considering the peak area of a standard nitrite concentration. The peak at 5.129 in the left chromatogram may or may not be nitrate, the peaks are not defined (see above). As any chromatographer will notice the presence of a huge front peak and the absence of a baseline, it is illegitime to use this peak for direct comparison with the nitrate peak in the right panel. The allegation that the recovery of nitrate from serum should be substantially lower than the reported 87%–90% based upon the presented argumentation of Tsikas' group does not make a valid point.

We are surprised about Tsikas and his problems with our study since he did not generate any experimental data by HPLC which contradicted our method. Based on our results we think that our method is an accurate, robust and easily applicable way for direct detection of nitrite and nitrate in serum samples.

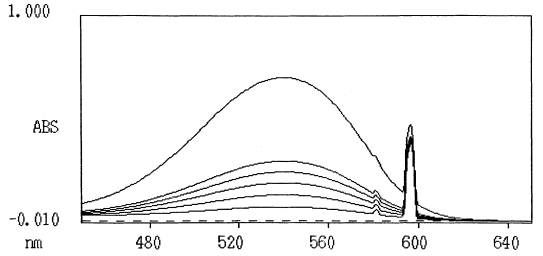

Fig. 4. Griess-assay: derivatisation of NO_2^- in water. From bottom to top: 1. 10 μM NO_2^-, 2 20 μM NO_2^-, 3. 30 μM NO_2^-, 4. 40 μM NO_2^-, 5. 50 μM NO_2^-, 6. 100 μM NO_2^-.

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