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Short communication

Measurement of serum nitrite/nitrate concentrations using high-performance liquid chromatography

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Abstract

Previous studies have reported increased serum concentrations of nitrite/nitrate – the degradation products of nitric oxide – in *Plasmodium vivax* malaria and uncomplicated *Plasmodium falciparum* malaria. In all these studies, however, nitrite/nitrate has been measured spectrometrically using Griess reagent which carries major disadvantages in the determination of serum nitrite/nitrate. The method does not allow an exact differentiation of nitrite and biogenic amines that are physiologically present in plasma. In the present study we introduce high-performance liquid chromatography as a new, accurate and cost effective method for determination of serum nitrite/nitrate levels. Significantly increased nitrate concentrations were found in malaria patients and serum values remained above normal levels for at least 21 days. It could be shown that our HPLC method is a sensitive and cost-effective method for direct determination of nitrite/nitrate in serum samples, which is not influenced by the presence of biogenic amines. © 1998 Elsevier Science B.V.

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1. Introduction

Nitric oxide is one of the ultimate effector molecules in the host defense against many intra- and extracellular pathogens [1]. Additionally, serum levels of nitrite and nitrate – the degradation products of nitric oxide – are increased in patients with *Plasmodium falciparum* and *Plasmodium vivax* malaria and may be indicators for the severity of disease [2,3].

The authors of previous studies used a spectrometrical method [4,5], with Griess reagent. As this method does not allow differentiation between nitrite/nitrate and biogenic amines physiologically present in plasma the accuracy of results may vary considerably. We have developed a sensitive and cost-effective method for direct determination of nitrite and nitrate using high-performance liquid chromatography (HPLC).

It was the aim of the present study to determine serum levels of nitrite and nitrate in patients with complicated *Plasmodium falciparum* malaria over

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the course of time to evaluate the performance of a novel HPLC method.

2. Experimental

2.1. Location of the study

The study was performed at the hospital for Tropical Diseases in Bangkok, Thailand.

Patients ($n=25$) with malaria met the following criteria: (1) age: 11–54 years, (2) infection with *Plasmodium falciparum* only during study period, (3) no application of chemotherapeutic drugs during the preceding 14 days and (4) symptoms of severe and complicated malaria defined by World Health Organisation (WHO) criteria [6]. There was no difference in dietary intake between the patients and healthy control group. The study was approved by the Ethical Board of the Mahidol University, Bangkok, Thailand and all patients gave informed consent before enrolment in the study. Fifteen healthy volunteers were used as control group.

2.2. Treatment

Malaria was treated with intravenously applied artesunate or artemisinin derivatives followed by mefloquine, the control group received no antimalarial treatment.

All patients were observed for 21 days. Blood samples for determination of serum nitrite/nitrate concentrations were taken on days 0, 7, 14 and 21.

2.3. Nitrite/nitrate HPLC

The chromatographic system consisted of a Shimadzu Sil-6B autoinjector port, a Shimadzu LC-9A pump, a UV–Vis detector SPD- 10 AV and a Shimadzu LC-workstation (all instruments Shimadzu, Tokyo, Japan). The column was an ion-exchanger based on styrene divinylbenzene which was modified in our laboratory with quaternary amine as follows: styrene divinylbenzene (10–20 μm particle size, 100 Å pore size, Hamilton, NV, USA) was modified into chloromethyl styrene divinylbenzene as described by Fritz et al. [7]. The displacement of chloride ion from chloromethyl styrene divinylbenzene was accomplished by shaking the resin with a solution of trimethylamine in anhydrous dimethylformamide (DMF) in the presence of sodium iodide at 100°C for 12 h. The resultant poly styrene divinylbenze-trimethyl ammonium resin was then poured into water, stirred for 30 min, filtered and washed with a 3 *M* solution of hydrochloric acid for 12 h. Filtration, followed by treatment with a dilute solution of 10% (w/w) sodium hydroxide for 20 h gave the desired resin which was filtered, successively washed several times with water and methanol and dried in vacuo at 70°C. The capacity was found to be 0.013 mequiv./g resin. The column was packed by slurring an appropriate weight of the resin in methanol and pumping the slurry into the column housing (150 mm×4.0 mm I.D.) at a flow-rate of 2 ml/min. The packed column was then washed with 150 ml of deionised water and 250 ml of the eluent prior to use in experiments. The mobile phase consisted of 10 m*M* methansulfonic acid sodium salts adjusted to pH 8.5 with 0.1 *M* NaOH. The flow-rate was 1 ml/min. The effluent was monitored at 214 nm.

Sample preparation was as follows: 1-ml serum was deproteinized with trifluoracetic acid, vortexed for 60 s and centrifuged at 7500 *g* for 5 min. Five-hundred μl of supernatant was added to 100 μl of 5% (w/w) NaOH solution and 400 μl of bidistilled water. The solution was then loaded onto a solid-phase extraction (SPE) column (C_{18}, 100 mg, Varian, Harbor City, CA, USA). The effluate was loaded again onto our developed solid-phase ion-exchange column. This was then washed with 2×1 ml of deionised water and finally eluted with 200 μl of 0.5 *M* solution of methansulfuonic acid sodium salts, adjusted to pH 8.5 with 0.1 *M* NaOH solution. Twenty μl was injected onto the HPLC system through the autoinjector. The sample stability at room temperature was at least 1 month.

Values are expressed as μmol/l, the minimal detectable concentration was 0.1 μmol/l. Because of the rapid reaction of nitric oxide (NO) with oxyhaemoglobin we predominantly detected nitrate in our serum samples.

Nitrite and nitrate concentrations are added and are expressed as nitrite/nitrate concentration.

The standard spectrometrical method was performed as described by Schmidt et al. [8]. In brief,

500 μl of nitrite free water was spiked with 1, 2, 5, 10, 50 and 100 nmol nitrite. Twenty-five μl sulfanilamide, 25 μl of 2.5 *M* HCl and 25 μl of 0.1% naphthylethylendiamin were added, and incubated at 21°C for 30 min. Additionally, the spectrum was measured at 546 nm by a photometer. In a second step different biological amines, which are physiologically present in plasma were added to the spiked solutions to evaluate interference of these substances with the nitrite measurement. The same procedure was performed in serum samples.

2.4. Statistical analysis

Data are expressed as mean and standard error of the mean and analyzed using ANOVA and Tukey-test. Spearmen correlation coefficients between different parameters were calculated. Probability values of $p<0.05$ were considered statistically significant.

3. Results and discussion

Typical chromatographic traces of nitrite and nitrate obtained after direct injection and extraction from biological sample are presented in Fig. 1. The limit of quantification in serum was defined as the lowest concentration of nitrite and nitrate resulting in a signal-to-noise ratio of 3:1. The lowest detection limit was 0.1 μmol/l.

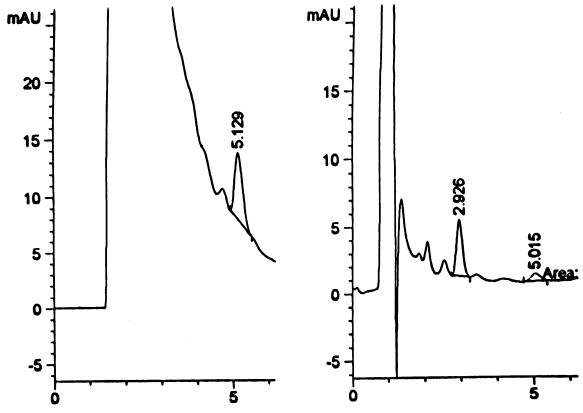

Fig. 1. Chromatogram of nitrite at 2.926 min and nitrate at 5.015 min from the same patient sample. Left panel after direct injection and right panel after SPE over a C_{18} column and ion-exchange cartridge as described in Section 2.3.

3.1. Recovery

Human serum from healthy volunteers was spiked with 0.1, 0.5, 1.0, 5.0 and 10.0 μmol/l of each nitrite and nitrate. Ten aliquots of each sample were chromatographed as described in Section 2.3 and the resulting peak areas were compared with the peak areas that resulting from aqueous solutions at the same concentration. The recovery amounts from sera were 87.7±6.8%, 89.9±4.7%, 90.0±3.2%, 91.6±4.7% and 92.8±3.6% – by coefficients of variation (C.V.) 5.9%, 5.4%, 6.0%, 4.8% and 5.1% for determination of 0.1, 0.5, 1.0, 5.0 and 10.0 μmol/l serum, respectively.

3.2. Precision

Human sera from healthy volunteers were spiked with known amounts of nitrite and nitrate.

3.3. Intra-day reproducibility

Ten aliquots of serum containing 0.1, 0.5, 1.0, 5.0 and 10.0 μmol/l of nitrite and nitrate, respectively, were randomly distributed in different series of assays on the same day. The intra-day reproducibility characterized by C.V. was 6.4%, 5.6%, 4.9%, 5.1% and 5.0% for assays of 0.1, 0.5, 1.0, 5.0 and 10.0 μmol/l, respectively.

3.4. Inter-day reproducibility

Ten aliquots of the same solution used for the within-day study were assayed one-by-one during 10 days, using each time the calibration curve of the day. The inter-day reproducibility precision values calculated by C.V. were 5.3%, 4.3%, 3.6%, 3.8% and 4.1% for assays of 0.1, 0.5, 1.0, 5.0 and 10.0 μmol/l, respectively.

In previous studies nitrite/nitrate was measured after exposing plasma to a copper–cadmium–zinc catalyst to convert nitrate to nitrite and then adding the effluent of the catalyst product to Griess reagent [4]. This procedure carries major disadvantages. Nitrite only is detected by the Griess reagent while nitrate has to be measured indirectly after its reduction to nitrite. It is not possible to predict how much of the sample's nitrate content is converted to nitrite

during the reductive process in the copper–cadmium–zinc catalyst. Additionally, the complex formation of nitrite and Griess reagent is a variable process and may influence the measured concentrations. As shown in our experiments the Griess reagent reacts with free biogenic amines other than nitrite. As these substances can be present in plasma under physiological conditions they may produce false positive results. Both nitrite and biological amines have the same spectrum of absorption making them impossible to differentiate by Griess reagent. Thus, the published concentrations measured by Griess reagent have to be considered as approximations, influenced by many factors. Comparison of nitrite/nitrate measurements using the Griess reagent and our HPLC method: in amine free solution spiked with nitrite both methods produced comparable results. In solutions containing biological amines nitrite concentrations determined by the Griess reagent were $1 \cdot 10^2$ to $1 \cdot 10^3$ higher than those measured by HPLC. The same differences were found in patient plasma samples. Furthermore, measuring nitric oxide with Griess reagent is a time consuming process. HPLC, which is a very sensitive, rapid and accurate method with a detection limit for nitrate of 0.1 μmol/l, carries non of these disadvantages. The degradation products of nitric oxide nitrite and nitrate are measured directly. Measurement of nitric oxide itself is complicated by its very short half life [9] and would therefore require on-line measurement which is unsuitable for routine use.

3.5. Clinical findings

The mean duration of fever before admission to the hospital was 3.7±1.8 days. The highest temperature prior to treatment was 40.0°C. The median parasite count prior to treatment was 313 060 (minimal/maximal parasite count: 546/4 000 070) asexual parasites/μl. The mean parasite clearance time was 60.51±25.97 h.

Serum nitrate concentrations in patients were significantly higher than in the control group at any time during the study ($p<0.01$).

The mean±S.E.M. nitrate/nitrite concentration was 12.01±2.18 (nitrite: 0.52±0.29) μmol/l at day 0, 12.25±1.75 (nitrite: 0.46±0.14) at day 7, 13.79±2.25 (nitrite: 0.23±0.07) at day 14, and finally 17.6±2.57 (nitrite: 0.15±0.05) at day 21.

In the healthy volunteers control group the sum of nitrite/nitrate concentration was 1.1±0.05 μmol/l.

There was a significant correlation both between the parasite-clearance time and the sum of serum nitrite/nitrate levels at day 7 ($p<0.018$) and the serum creatinine-concentrations and the sum of serum nitrite/nitrate levels ($p<0.027$) at day 0.

Nitric oxide is produced in large quantities during host defense and immunologic reactions having both anti-inflammatory and pro-inflammatory properties [10].

In the present study we found increased levels of nitrite and nitrate in patients with complicated *Plasmodium falciparum* malaria for at least 21 days, indicating activation of the nonspecific immunity. This finding is in accordance with Kremsner et al. [11] who found increased nitrite/nitrate levels for 28 days.

4. Conclusions

Summarizing the results our HPLC protocol provides an excellent method to measure the degradation products of nitric oxide as a basis for accurate estimates of nitric oxide concentrations. To our knowledge HPLC has not been used before to measure serum concentrations of nitrite and nitrate in humans. In our hands it has proved a sensitive, accurate and reproducible method.

We found increased serum concentrations of nitrate/nitrite in patients with complicated *Plasmodium falciparum* malaria for at least 21 days which seems to play an important role in the host defense of malaria.

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References

[1] K.A. Rockett, M.M. Awburn, W.B. Cowden, I.A. Clark, Infect. Immun. 59 (1991) 3280.
[2] I.A. Clark, K.A. Rockett, W.B. Cowden, Lancet 340 (1992) 894.
[3] S. Cot, P. Ringwald, B. Mulder, P. Miailhes, J. Yap Yap, A.K. Nussler, W.M. Eling, J. Infect. Dis. 169 (1994) 1417.
[4] P. Jacobs, D. Radzioch, M.M. Stevenson, J. Immunol. 155 (1995) 5306.
[5] S.S. Greenberg, J. Xie, J.J. Spitzer, J.F. Wang, J. Lancaster, M.B. Grisham, D.R. Powers, T.D. Giles, Life Sci. 57 (1995) 1949.
[6] D.A. Warrell, M.E. Molyneux, P.F. Beales, T. Roy. Soc. Trop. Med. H. 84(Suppl. 2) (1990) 1.
[7] J.S. Fritz, D.T. Gjerde and C. Pohlandt, Ion Chromatography, Dr. Alfred Hüthig Verlag, Heidelberg, Basel, New York, 1982, p. 102.
[8] H.H. Schmidt, T.D. Warner, M. Nakane, U. Forstermann, F. Murad, Mol. Pharmacol. 41 (1992) 615.
[9] R.M. Palmer, A.G. Ferrige, S. Moncada, Nature 327 (1987) 524.
[10] E. Moilanen, H. Vapaatalo, Ann. Med. 27 (1995) 359.
[11] P.G. Kremsner, S. Winkler, E. Wildling, J. Prada, U. Bienzle, W. Graninger, A.K. Nussler, T. Roy. Soc. Trop. Med. H. 90 (1996) 44.