Amino Acids (2003) 24: 375–381
DOI 10.1007/s00726-002-0346-0

Amino
Acids
Printed in Austria

The low nanomolar levels of N^G-monomethylarginine in serum and urine of patients with chronic renal insufficiency are not significantly different from control levels

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Received April 3, 2002
Accepted October 7, 2002
Published online January 20, 2003;

Summary. There are no reliable mean values of N^G-monomethylarginine (NMMA) in blood and urine of patients with renal insufficiency available in the literature. Therefore we investigate whether the NMMA levels are changed in blood and urinary excretion of nondialysed and dialysed patients with chronic renal insufficiency to evaluate whether NMMA may reach sufficiently increased concentrations in blood of the patients to exert toxic biological activity.

In nondialysed as well as in dialysed patients we find no significant difference in serum concentration of NMMA between patients and controls. In nondialysed patients (all with a residual creatinine clearance lower than 15 ml/min), we find 94.5 ± 26.1 nM (mean ± SD) versus 94.6 ± 19.5 nM in controls. Similar levels are found in serum of haemodialysed patients (each with serum creatinine levels >700 μM): 83.0 ± 20.2 nM. The urinary excretion of NMMA in nondialysed patients is also not significantly different from the excretion of controls: 123 ± 110 in patients versus 157 ± 117 nmol/24 hrs in controls. Furthermore, the clearance of NMMA is much lower compared to the clearance of the dimethylarginine derivatives.

Based on the literature, the low nanomolar levels of NMMA found in blood of patients with renal insufficiency do not support the statement that NMMA proper may act as a uremic toxin.

Keywords: N^G-monomethylarginine – Guanidino compounds – Methylated arginine residues – Renal insufficiency – Uraemia – Nitric oxide (NO) – Nitric oxide synthase (NOS)

Introduction

Catabolism of proteins containing methylated arginine residues releases free methylarginine residues including NMMA, asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA). NMMA and ADMA are equipotent endogenous inhibitors of nitric oxide (NO) synthase (NOS), which catalyses the formation of NO and citrulline from arginine. In contrast, SDMA does not inhibit NOS activity (Vallence et al., 1992).

NO is an important vasodilator, inhibitor of platelet aggregation, neuromodulator and plays a role in immune function (Moncada and Higgs, 1993; Moncada and Higgs, 1995; Bredt and Snyder, 1994; Reyes et al., 1994; Blantz et al., 2000). Disturbed NO synthesis has been suggested in patients with chronic renal failure and changes in NO levels could contribute to some of the symptoms observed in these patients (Noris et al., 1993; Blantz et al., 1996; MacAllister et al., 1996 and 1998; Schmidt et al., 1999; Aiello et al., 1999).

Renal clearance and metabolism are important mechanisms for elimination of the methylated arginine derivatives and accumulation of these residues has been suggested to contribute to the uremic symptomatology (MacAllister et al., 1996 and 1998; Mendes Ribeiro et al., 1996 and 2001; Segarra et al., 1999).

Mean levels of NMMA in blood of patients with renal insufficiency are scanty. Mendes Ribeiro et al. (1996) published mean plasma levels of 14 μM in haemodialysed uremic patients. Although not giving mean values, Anderstam et al. (1997) suggested,

however, that there was no difference in NMMA levels between controls and dialysed patients; they roughly estimated the absolute levels between 10 and 100 nM. Levels in nondialysed patients have not been reported. When the circulating NMMA levels are indeed up to 14 μM, NOS activity can be inhibited leading to vasoconstriction (MacAllister et al., 1994).

In this study we investigate whether the NMMA levels are changed in blood and urinary excretion of nondialysed and dialysed patients with chronic renal insufficiency to evaluate whether NMMA may exert toxic biological activity at the concentration found in blood of these patients.

Subjects and methods

Patients

This study considers 20 nondialysed patients with chronic renal insufficiency (10 men and 10 women) and a residual creatinine clearance <15 ml/min (7.8 ± 3.8). Ages range between 24 and 81 years, serum urea concentrations between 16 to 46 mM and serum creatinine between 340 and 1,262 μM. Etiological diagnoses are as follows: tubulo-interstitial nephritis (n = 5), diabetic nephropathy (n = 4), nephrangiosclerosis (n = 3), glomerulonephritis (n = 2), polycystic kidney disease (n = 2), focal glomerulosclerosis (n = 1), Alport syndrome (n = 1), renal artery stenosis (n = 1) and kidney failure of unknown origin (n = 1). Diagnoses are based on medical history, clinical findings, biochemical data, radiological examinations and if appropriate on histological data. Renal function in this group is estimated by calculating creatinine clearance from creatinine determinations on serum and 24 hour urine collections.

This study also considers 21 patients treated by regular haemodialysis (11 men and 10 women), with serum creatinine levels >700 μM. Ages range from 25 to 83 years, serum urea from 15 to 41 mM and serum creatinine from 703 to 1,071 μM. Etiological diagnoses in this second group are: tubulo-interstitial nephritis (n = 3), diabetic nephropathy (n = 2), nephrangiosclerosis (n = 2), glomerulonephritis (n = 2), polycystic kidney disease (n = 3), nephrolithiasis (n = 2), pyelonephritis (n = 1), IgA nephropathy (n = 2), bilateral nephrectomy due to malignancy (n = 1), obstructive nephropathy (n = 1), amyloidosis (n = 1) and kidney failure of unknown origin (n = 1). Twelve patients of the second group were anuric.

Conventional haemodialysis was performed 3 times 4 hours per week with low flux polysulfone (F8, Fresenius Medical Care, Bad Homburg, Germany). A dialyzer blood flow (Q_B) of 300 mL/min and a dialysate flow (Q_D) of 500 mL/min were maintained. Kt/V was targeted at >1.4.

Age- and sex-matched control samples are obtained from healthy volunteers and individuals presenting with transient neurological complaints in whom, after performing clinical and chemical diagnostic tests, no neurologic, renal, hepatic or metabolic disease was diagnosed. Serum urea of controls range from 2.4 to 8.6 mM and serum creatinine from 44 to 96 μM. There was no significant difference between the body weight of controls and the studied patients with renal insufficiency.

The study is approved by the local ethics committee and all subjects gave their written informed consent.

Collection and preparation of samples

Fasting morning blood is taken from all studied subjects. From the control subjects and the nondialysed patients, a corresponding 24-hour urine collection is obtained as well. Blood from the dialysed patients are sampled just before the start of a haemodialysis session. After clotting, blood is centrifuged at 1,000 × g at 4°C for 10 minutes. A portion of the serum is analysed separately for urea as described earlier by Ceriotti (1971). The remaining serum is stored at −75°C until analysis.

For determination of creatinine and other guanidino compounds {such as guanidinosuccinic acid (GSA), guanidinoacetic acid (GAA), arginine (ARG) and creatine (CT)} in serum and urine, one sample volume is vortex-mixed with one volume of a 200 g/L trichloroacetic acid solution. The protein complexes are precipitated after centrifugation during 10 minutes at 4°C and 15,850 × g (Beckman Instruments, Inc, Palo Alto, California 94304, USA). One volume deproteinised serum supernatant is diluted 20 times with sample buffer (200 mM tri-sodium citrate dihydrate; pH 2.5); one volume deproteinised urine supernatant is diluted 40 times. Two hundred μL of the diluted solutions are injected automatically on the column.

For determination of NMMA in serum and urine 200 μL sample volume is vortex-mixed with 50 μL of a 100 g/L sulfosalicylic acid solution. Again, protein complexes are precipitated after centrifugation during 10 minutes at 4°C and 15,850 × g (Beckman Instruments, Inc, Palo Alto, California 94304, USA). One volume deproteinised serum supernatant is diluted with one volume sample buffer (150 mM tri-sodium citrate dihydrate; pH 2.5) and one volume deproteinised urine supernatant is diluted 4 times. Two hundred μL of the diluted solutions are injected automatically on the column.

Laboratory methods

For determination of creatinine levels, a Biotronic LC 6001 (Biotronik, Maintal, Germany) amino acid analyser adapted for determination of guanidino compounds (including creatinine) is used. Creatinine and the other guanidino compounds are separated over a cation exchange column using sodium citrate buffers and are detected with the fluorescence ninhydrin method as previously reported in detail (Marescau et al., 1985 and 1986). A Jasco (Jasco Internation Co, Tokyo, Japan) Model FP-920 fluorescence detector is used at 395 nm and 500 nm for Ex. and Em. respectively.

NMMA levels are determined using a Biotronic LC 5001 amino acid analyser: separation of amino acids and analogues occur on a cation exchange resin (BTC 2710; Biotronik, Maintal, Germany) and detection is done with postcolumn derivatisation using orthophthaldialdehyde (OPA) reagens.

A resin-height of 240 mm into a column with 3.2 mm internal diameter is used; the temperature of the column is 60°C for determination in urine and 65°C for serum. The buffer flow is 240 μL/min. Amino acids and NMMA are eluted with a 150 mM tri-sodium citrate dihydrate buffer (pH 3.7) during 213 min. The resin is washed with 0.70 M NaOH for 40 min. Thereafter the resin is re-equilibrated with a 150 mM tri-sodium citrate dihydrate buffer (pH 3.0) for 56 min. After the re-equilibration period, the flow of buffer and OPA is stopped 5 min to omit death-volume on the top of the resin. The time between two injections (automatically with cooled sample injector) is 314 min. A Jasco Model FP-920 fluorescence detector is used at 350 nm and 430 nm for Ex. and Em. respectively.

Chemicals

OPA-reagens: 1 M Boric acid is made under slowly mixing and bubbling with nitrogen gas; degassed HPLC water is used. The

vessel is placed in chilled ice water since 10 g KOH pellets is added 5 times to the mixture. All the boric acid is dissolved after approximately 3 hours. A solution of 0.5 g phtaldialdehyde in 10 mL methanol is then added to the solution, which is filtered afterwards. Finally 2 mL mercapto-ethanol is added. The reagens is pumped through the analytical system with a flow rate of 132 μL/min.

Chemicals for buffer and reagent preparation are of analytical grade and obtained from Merck (Darmstadt, Germany). Standard creatinine is obtained from Merck, NMMA and the other guanidino compounds from Sigma Chemical (St Louis, MO, USA).

Calculation of clearance of NMMA and other guanidino compounds

Endogenous clearance rates are only calculated for those compounds with detectable levels as well in serum as in urine for all studied controls and patients. The clearances are calculated taking into account the levels in 24 hour urine collections and the levels in serum and using the classical formula (Al Banchaabouchi et al., 2001).

Statistics

The results are presented as mean ± standard deviation (SD). Results from the nondialysed and dialysed patients and their age- and sex-matched controls are compared with Students T test.

Results

Under the applied elution conditions, the retention time of serum NMMA is 162 min, that of urinary NMMA 172 min (Fig. 1 and 2). Different column temperatures are used for determination of NMMA in serum (65°C) and urine (60°C): using a higher temperature (65°C) gives a better resolution between NMMA and arginine. However in the urinary elution chromatogram of control subjects, run at 65°C, NMMA is eluting on a tailing peak (X) (Fig. 2) of one or more, more acidic amino acids which are present at much higher levels than NMMA. The peak (X) is much smaller in urine of patients with renal insufficiency compared to controls. To obtain a higher resolution between peak X and NMMA in urine, the samples are analysed with a column temperature of 60°C and since the concentration of arginine in urine is mostly (controls and patients) lower than the concentration in the corresponding serum, the resolution between NMMA and arginine is not disturbed. The identity of NMMA in serum and urine is suggested after spiking with standard NMMA (Fig. 1B and 2B). Other amino acids and methylated amino acids, present in physiological fluids, like carnosine, homocarnosine, tryptophan and methylated histidine and lysine derivatives elute before NMMA.

The chromatographic characteristics of the used procedure are also tested: the detection limit, considering two times the noise level as being no peak, is 2 pmol injected absolutely on the column in an injection volume of 200 μL which corresponds to 10 nM for an injection volume of 200 μL. The % recovery of NMMA has been calculated after adding known amounts of NMMA standard to control human urine (n = 6): range = 97.6 − 105% (mean ± SD = 100.9 ± 3.08). The coefficient of variation on the reproducibility has been calculated on 12 successive analyses of standard NMMA: cv = 3,9% (within run precision). Between run precision was calculated on 20 analyses on different days over a 2-month period : cv = 5.1%.

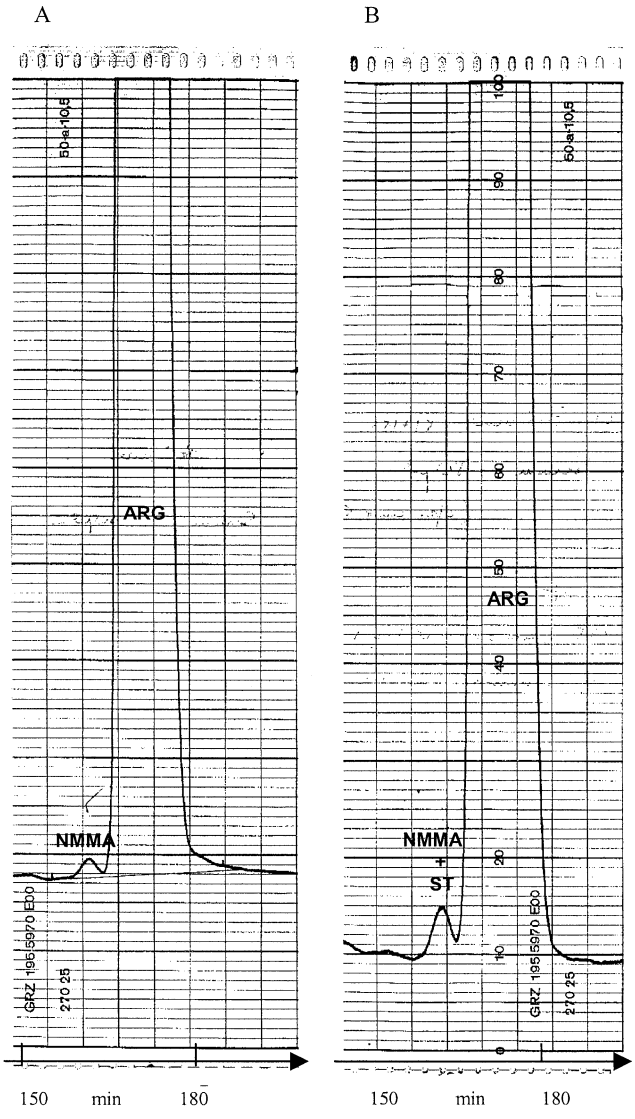

Fig. 1. Chromatographic raw data showing a fraction of an analysis of human serum analysed at 65°C: A represents native serum, B the same serum spiked with standard NMMA

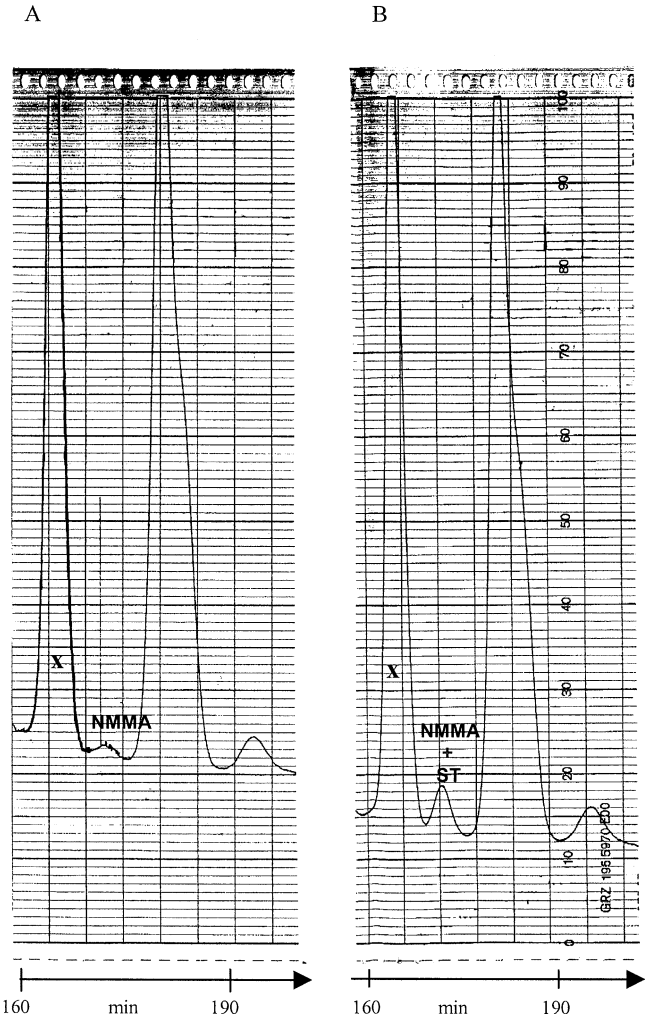

Fig. 2. Chromatographic raw data showing a fraction of an analysis of human urine analysed at 60°C: **A** represents native urine, **B** the same urine spiked with standard NMMA

Table 1 enlists mean and SD of NMMA and creatinine concentrations in serum and urinary excretion of nondialysed patients with chronic renal insufficiency with a residual creatinine clearance lower than 15 ml/min. No significant difference in concentration of NMMA in serum ($p = 0.995$) and urine ($p = 0.607$ for expression per 24 hrs; $p = 0.638$ for expression per g CTN; $p = 0.153$ for expression in nM) between the nondialysed group and their corresponding age- and sex-matched controls was found. If we subdivide the nondialysed patients with chronic renal insufficiency into two groups, with residual creatinine clearance lower ($n = 12$) and higher ($n = 8$) than 8 ml/min, also no significant difference in concentration of NMMA in serum and urine between controls and patients was found for the two subgroups.

Similar results have been found in serum of dialysed patients with serum creatinine levels higher than 700 μM (Table 2): no significant difference in concentration of NMMA in serum between the dialysed group and their corresponding age- and sex-matched controls was found ($p = 0.076$). Again, if we subdivide the dialysed patients with chronic renal insufficiency into two groups, with serum creatinine levels lower ($n = 15$) and higher ($n = 6$) than 900 μM, also no significant difference in concentration of NMMA in serum between controls and patients was found for the two subgroups.

Table 3 shows the endogenous clearances of NMMA, CTN and other guanidino compounds in controls and nondialysed patients with chronic renal insufficiency with a residual creatinine clearance <15 ml/min. As we observed before, some guanidino compounds such as guanidinosuccinic and guanidinoacetic acid, ADMA and SDMA are cleared very well by the healthy kidneys and their clearance is comparable to the clearance of creatinine, another guanidino compound (Marescau et al., 1997). Arginine, also a guanidino compound, is not cleared at all. Creatine and also NMMA are cleared from the body to a higher degree than arginine. However the clearance of NMMA is significantly lower than the one of creatine and significantly higher than the one of arginine.

In nondialysed patients the clearance of the studied products is lower than in controls except for NMMA and arginine.

Discussion

Our first aim was to present an analytical method for the quantification of precise levels of methylated arginine derivatives (NMMA, ADMA and SDMA) in one single run using cation exchange resin for separation of non derivatised amino acids. This was no problem when analysing standard solutions of amino acids and substituted amino acids, present in physiological fluids, with similar concentration levels. However, when analysing physiological fluids, additional resolution problems arose: e.g., our results showed that NMMA levels in serum of controls were almost 1,000 times lower than the levels of arginine eluting just after NMMA. A good resolution of NMMA and arginine required specific demands (column temperature) which influence the chromatographic characteristics of ADMA and SDMA so that we were unable to present precise values of the three

Table 1. Serum and urinary excretion levels of NMMA and CTN in controls and nondialysed patients with chronic renal insufficiency (mean ± SD)

	serum			
	Controls (n = 20) (mean Cl_{CTN} = 106.2 ± 52.5 ml/min)		Patients (n = 20) (mean Cl_{CTN} = 7.8 ± 3.8 ml/min)	
	mean	SD	mean	SD
NMMA (nM)	94.6	19.5	94.5	26.1
CTN (μM)	65.6	14.1	645.7	286.7
	urine			
	Controls (n = 20) (mean Cl_{CTN} = 106.2 ± 52.5 ml/min)		Patients (n = 20) (mean Cl_{CTN} = 7.8 ± 3.8 ml/min)	
	mean	SD	mean	SD
NMMA (nmol/24 hrs)	157	117	123	110
CTN (μmol/24 hrs)	10,100	6,253	6,544	3,345
NMMA (nM)	142	123	96.9	63.6
CTN (μM)	8,286	3,774	5,210	1,759
NMMA (nmol/g CTN)	158	147	179	133

The urinary excretion is expressed per 24 hours and per gram creatinine. The urinary concentration is also given

Table 2. Levels of NMMA and creatinine (CTN) in serum of haemodialysed patients with chronic renal insufficiency (mean ± SD)

	Controls (n = 21)		Patients (n = 21)	
	mean	SD	mean	SD
NMMA (nM)	93.7	18.2	83.0	20.2
CTN (μM)	64.5	13.0	847	109

Table 3. Endogenous clearance (ml/min) of NMMA, CTN and other guanidino compounds in controls and nondialysed patients with chronic renal insufficiency with a residual CTN clearance <15 ml/min

	Controls		Nondialysed Patients	
	mean	SD	mean	SD
NMMA	1.12	0.79	1.02	1.03
CTN	106	52.5	7.82	3.80
GSA	70.0	44.9	5.38	2.46
GAA	115	81.8	6.91	4.14
ARG	0.12	0.12	0.11	0.13
CT	18.7	24.5	2.01	1.27
ADMA(*)	77.5	27.8	7.35	5.24
SDMA(*)	80.1	31.1	5.75	2.95

(*) the clearances of ADMA and SDMA are those published by Marescau et al. (1997): controls (n = 33); nondialysed patients (n = 18, Cl_{CTN} <10 mL/min.)

methylated arginine derivatives in physiological fluids after one single analytical run using the method described here. The absolute levels of ADMA and SDMA in serum of control subjects using the above mentioned method were almost twice the ones found using another analytical method for the specific determination of the dimethylarginine residues, described earlier (Anderstam et al., 1997; Marescau et al., 1997).

Mean levels of NMMA in blood of controls and patients with renal insufficiency are very scanty. Difficulties in determination by liquid chromatography and low endogenous levels can most likely be the cause. As Anderstam et al. (1997) mentioned themselves only "rough estimations" (10–100 nM) could be reported in controls as well as in patients. Meyer et al. (1997) present mean levels of 100 nM only in healthy controls, almost identical to the mean values found in our controls. Since the detection limit of the system used by Mendes Ribeiro et al. (1996) was around 2,000 nM for NMMA they could not present normal values, however they found increased levels of NMMA (mean levels of 14 μM) in plasma of end-stage renal failure patients on regular haemodialysis. These are, to our knowledge, the only published mean values of NMMA in blood of patients with renal failure. Our results demonstrate much lower levels of NMMA in

blood of patients with renal insufficiency. Furthermore we observed no significant difference between the NMMA levels found in serum of controls and patients with renal failure. Therefore the suggestion (Mendes Ribeiro et al., 2001) that in renal failure intracellular supply of arginine may be limited due to competition for transport by NMMA and arginine can be questioned. The influence of low nanomolar (100 nM) levels of NMMA on NOS activity will be also negligible (MacAllister et al., 1994).

Twenty-four hour urinary excretion levels of NMMA from adult man are difficult to find in the literature. To our knowledge, this report is the first giving urinary excretion levels of NMMA in adult controls and patients with renal insufficiency. We observed no significant difference between the 24 hours urinary excretion levels of NMMA found in controls and nondialysed patients with renal failure. The clearance of NMMA clearly shows that the renal removal of NMMA cannot be considered to be similar to that of other guanidino compounds such as guanidinosuccinic and guanidinoacetic acid or ADMA and SDMA. Further investigations will be needed to show whether NMMA is fully or partially reabsorbed at the level of the tubuli.

In conclusion, the mean levels of NMMA in blood of controls, haemodialysed and nondialysed patients with renal failure are around 100 nM and are not significantly different. The mean urinary excretion levels of NMMA in nondialysed patients with renal failure ($\cong$ 120 nmol/24 hours) are also not significantly different from controls. Today, with the existing literature, the low nanomolar levels of NMMA found in blood of patients with renal insufficiency do not support the statement that NMMA exerts toxic biological activity and or that NMMA is a uremic toxin.

Acknowledgements

We thank the University of Antwerp, FWO (Fonds voor Wetenschappelijk Onderzoek) (G.0027.97 and G.0394.00), the Born-Bunge Foundation, the OCMW Medical Research Foundation and Neurosearch Antwerp for the financial support.

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