Innovare Academic Sciences

International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 6, Issue 5, 2014

Original Article

SPECTROFLOURIMETRIC MEASUREMENT OF NITRIC OXIDE IN MICE PLASMA, URINE AND KIDNEY HOMOGENATE IN DIABETIC NEPHROPATHY

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Received: 28 Mar 2014 Revised and Accepted: 01 May 2014

ABSTRACT

Objective: Nitric oxide (NO) plays a critical role in the pathophysiology of diabetic nephropathy. The reduction in NO levels observed in diabetic nephropathy cannot be accurately measured by Griess reaction. Present study aimed to determine the levels of the stable end products of NO, nitrate and nitrite after eight weeks of streptozotocin induced diabetes by flourimetric method.

Methods: The determination of NO concentration by flourimetric method was based on the reaction between 2, 3-diaminonaphthalene and NO to yield the highly flourescent 2, 3-naphthotriazole. Nitrate reductase and NADPH regenerating system was used to quantitate NO in protein precipitated plasma, urine and kidney homogenate after reduction of nitrate. The optimum conditions, such as flourimetric determination conditions (λex= 365 nm, λem= 415 nm), the influence of pH, and reaction time on the flourescence was examined.

Results: The flourimetric intensity was linear with the NO concentration in the range of 10-440 ng/ml with a R^2 of 0.998 (p< 0.0001). The limit of detection was 14.44ng/ml and the limit of quantitation was 43.76ng/ml. The intraday and interday precision and accuracy of the method were within the stipulated limit. The validated method was used to measure NO in the plasma, kidney homogenate and urine. The results showed a sharp decrease in NO levels.

Conclusion: The method is reproducible, sensitive and reliable. Owing to great pathological implications of NO in diabetes; the work may help to investigate underlying mechanism of diabetic nephropathy.

Keywords: Nitric oxide, Spectroflourimetric, Diabetic nephropathy, 2, 3-Diaminonaphthalene, Naphthotriazole.

INTRODUCTION

Nitric oxide (NO) is an important mediator of both physiological and pathophysiological processes [1]. The interest in measuring NO production has, not surprisingly, received enormous attention recently. Different methods for determining NO or its metabolites, nitrite and nitrate have been reported [2-4]. The abnormalities of renal NO generation have been linked to pathogenesis of renal disease in diabetes [5, 6].The contrasting state of NO have been reported in pathogenesis of diabetic nephropathy(DN). In the early phases of DN, increase in NO production and upregulation of NOS (Nitric Oxide Synthase) isoform expression have been reported. In contrast, a progressive decline in NO production and specifically NO bioavailability have been reported in the kidney with advancing renal failure [7]. The plasma, urine and renal NO levels have been found to reduces in diabetic nephropathy.

Because free NO is a transient species with a half-life of about 5 seconds, many investigations of this gaseous molecule have relied largely on studies of NOS. NO rapidly decomposes into stable end products, nitrite and nitrate. Quantification of nitrite and nitrate in biological fluids and tissues provides a useful method of indirectly estimating endogenously produced NO. It is known that biological fluids such as plasma, cerebrospinal fluid, and urine have relatively large amounts of NO which can be detected by Griess reaction. However, reduced bioavailability of NO is a very well-known phenomenon in diabetes; induced by hyperglycaemia mediated oxidative stress. In diabetic nephropathy urinary and kidney tissue nitrite levels decrease by 10-20 fold [8, 9]. The method of measuring nitrite by Griess reaction does not have sufficient sensitivity for such a low levels in diabetic nephropathy. Hence, a sensitive assay is needed to assess kidney tissue NO levels. Two methods based on sensitive flourescence measurements using 4-hydroxycoumarin or 2,3-di- amino naphthalene(DAN) have been reported [10, 11]. The flourescent product 3-amino-4-hydroxycoumarin is unstable under the assay conditions, unless the reaction is conducted at 0ºC [12]. The hydroxyl coumarin method applied to cerebral tissue measurements [13]. The author determined only nitrite levels in unfiltered crude tissue extracts. We selected the DAN method due to the stability of the flourescent product.

MATERIALS AND METHODS

Instruments and Reagents

The instruments used in this work are as follows: Flourescence spectrophotometer (LS 55 Flourescence spectrometer, PerkinElmer), Automatic Analytical Balance (Shimadzu AUX 220, Japan). Streptozotocin (STZ) and nitrate reductase were purchased from Sigma Chemical Co., Milwaukee, WI, USA. 2, 3-diaminonaphthalene (DAN), sodium nitrite, and nicotinamide adenine dinucleotide phosphate (NADP) were purchased from Himedia laboratories, Mumbai India. Trichloro acetic acid was procured from Loba Chemie, Mumbai, India. All other chemicals used were of analytical grade.

Methods

Sodium nitrite standard solution

The stock solution of sodium nitrite was prepared by dissolving 100mg $NaNO_2$ in 100 ml double distilled water. The appropriate volume of the stock was diluted to make the solutions in the concentration range of 10-440 ng/ml. All the solutions were kept in dark till the analysis was completed.

Preparation of stock solution of DAN

The stock solution of DAN was prepared by dissolving 10mg of DAN in 10 ml of double distilled water. This solution was kept in the dark at –21 °C and used when required with further dilution.

Flourimetric detection conditions

The flourescence spectra were scanned with scan speed of 1000 nm min^{-1} at λ_{ex} of 365 nm and λ_{em} of 415 nm. The slit width was adjusted to 2.5 nm.

Induction of diabetic nephropathy

All animal experiments were conducted in accordance with the guidelines of CPCSEA-India with prior approval from Institutional Animal Ethics committee (Protocol No. RPCP/IAEC/2012-2013/R-18). One week after the acclimatization animals were randomly assigned to either STZ or vehicle. The low dose STZ protocol described by Animal Models of Diabetic Complications Consortium (AMDCC) was followed for induction of diabetes[14]. In brief, mice were fasted prior to injection for 4 hours. A single intraperitoneal STZ (50mg/kg) injection was administered to each mouse for 5 days consecutively (n=6). Mice were supplied with 10% sucrose water to avoid sudden hypoglycemia post injection. A normal control group of mice were injected with 400 μL vehicle (sodium citrate buffer). Body weight and blood glucose (SD CHECK™ GOLD Blood Glucose Meter, SD Biosensor, Korea) were monitored 1 week after STZ injection and every week thereafter. Mice with blood glucose levels of >300 mg/dL were considered to be diabetic. After 4 weeks of induction of diabetes, urine and blood samples were collected every week to monitor onset of protein urea and to assess renal function parameters.

Sample preparation

Plasma

Blood was drawn from retro orbital tract; sodium citrate (3.8%) was used as anticoagulant. The collected blood was centrifuged at 4000 rpm at 4º C for 5 min and plasma was separated. Since even 10 uM haemoglobin completely abolishes the 2, 3-diaminonaphthotriazole signal [10], the plasma samples were treated with trichloroacetic acid to precipitate protein. The resultant solution was centrifuged and supernatant was used to assay NO levels.

Urine

Mice were housed in metabolic cages for collection of 24 hours urine samples. Urine was centrifuged at 10000 rpm to remove any contaminant substances. The setting up of cages and collecting samples were carried out between 15:00 and 16:00 to avoid food derived creatinine interference.

Kidney homogenate

After 10 weeks of STZ administration, all the animals were sacrificed and whole kidneys were removed. Kidney tissues were washed with cold phosphate buffered saline and homogenized (10%w/v) in ice bath. The homogenate was then centrifuged at 20000 rpm at 4°C for 20 min. The supernatant was used in analysis.

Assay procedure

Nitrite levels were determined by measuring the flourescence of 2, 3-diaminonaphthotriazole (NAT) (Fig.1). NO was determined following the reduction of nitrate to nitrite using nitrate reductase and NADPH [15, 16]. All the samples (volume as depicted in Table 1) were treated with 10ul of nitrate reductase (0.5U/ml) and 10ul of 0.05mM NADPH. The samples were then incubated at room temperature for 60 min. Following incubation, 20ul of DAN (0.05 mg/ml), 130 ul HCl (1.5N) were added. After 10 minutes, the reaction was stopped by addition of 130ul of NaOH (2N).The resultant solution was diluted to 2ml with double distilled water and the emission scan was recorded. Plasma and tissue NO levels were expressed as ng/ml, and urine NO levels were expressed as pg/ml.

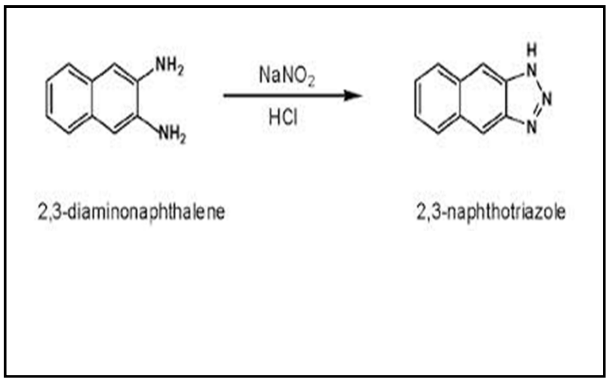

Fig. 1: Reaction between 2, 3-Diaminonaphthalene and nitrite

Table 1: Sample volume

Sample	Sample volume(ul)
Plasma	20
Urine	20
Homogenate	100

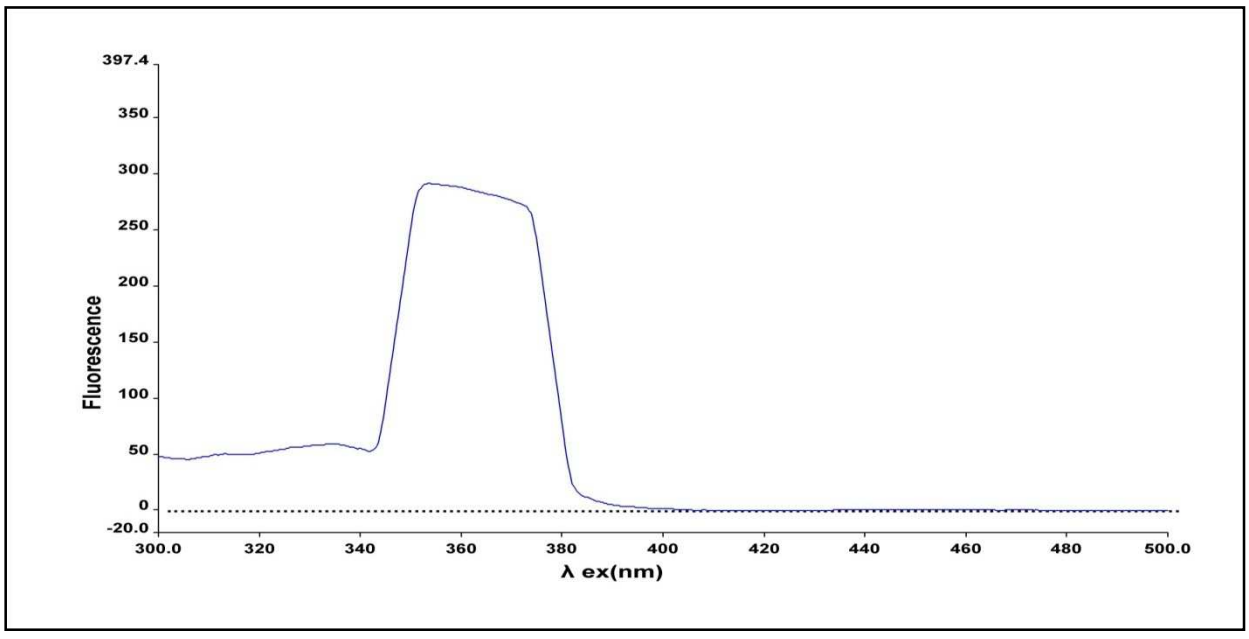

Fig. 2: Flourescence excitation spectra at λ emission = 415

Statistical analysis

All the measurements were expressed as mean ±S.D. Data were analyzed using a student *t*- test to compare control and diabetes group. A value of p <0.05 was considered significant.

RESULTS AND DISCUSSION

Optimization of flourimetric conditions:

Selection of analytical wavelengths

Sodium nitrate standards were incubated with freshly prepared DAN to form the flourescent product NAT as described in section 2. The emission spectra of sodium nitrite show maximum emission at 415 nm after excitation at 365 nm. So, 365 nm and 415 nm were selected as excitation wavelength and emission wavelength, respectively (Fig.2 and Fig.3a).

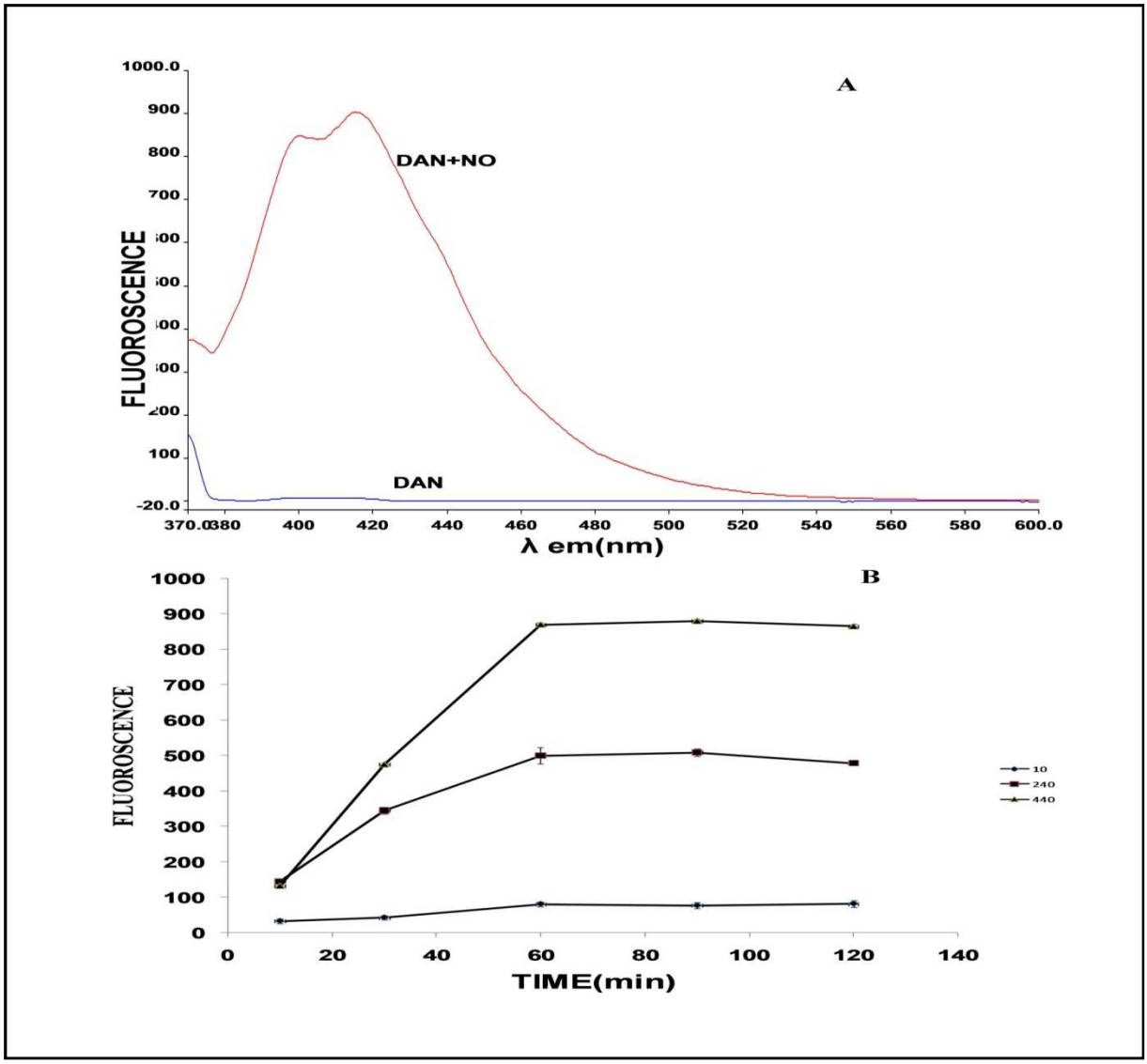

Fig. 3: Flourescence emission spectra at λ excitation = 365 Maximal emission peaks were at 400 and 415 nm at alkaline pH (A). No emission was observed at neutral or acid pH. B represents time dependence of flourescence development. 10, 240,440 ng/ml nitrite solutions were incubated with 2, 3-diaminonaphthalene at different time values and flourescence was measured at415 nm. After 60 min, flourescence was constant. Each data point represents the mean of measurements performed in triplicate.

pH Selection

It was observed that the flourescence intensity increased with increasing pH. Based on this, the final pH was adjusted to 11-12 with NaOH. At alkaline pH values, two major emission peaks were observed, one at 400 and other at 415 nm (Fig. 3a) with high flourescence intensity.

Selection of reaction time

To determine the time required to develop a constant flourescence, 10,240 and 440ng/ml nitrite solution were incubated with DAN for 10, 30, 60, 90,120 min. This reaction requires an acidic medium; thus, alkalization after the reaction is required to measure flourescence. Fig. 3b shows that an incubation time of 60 min was enough for the reaction with DAN to be completed.

Analytical method validation

The method validation was carried out as per ICH Q_2 R_1 guide lines. Samples were incubated with nitrate reductase for 60 min using 0.05mM NADPH and then reacted with DAN.

Linearity and range

The linear regression data for the calibration curves (n=5) shows a good linear relationship over the concentration range of 10-440ng/ml (Fig. 4).

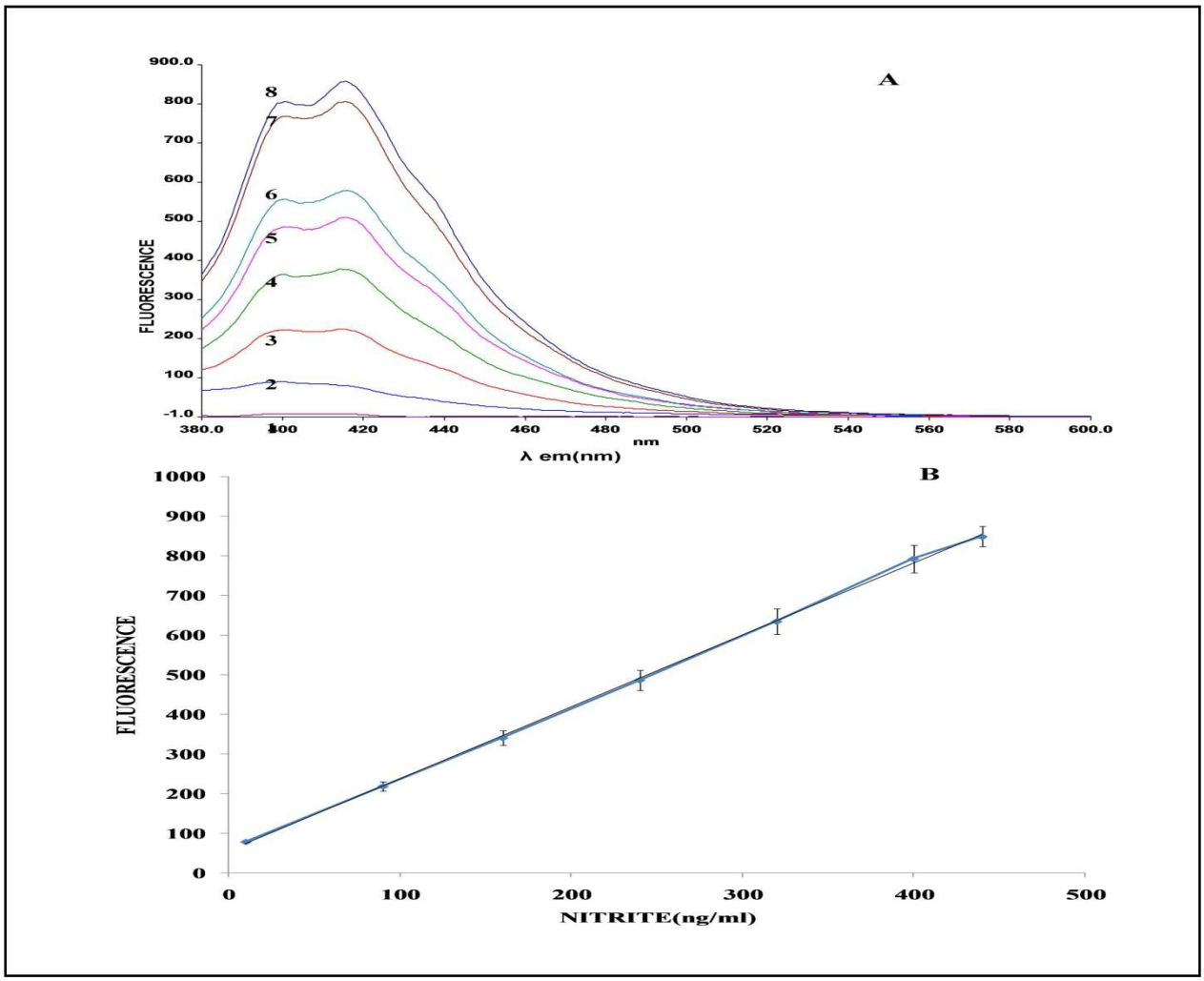

Fig. 4: Flourescence emission overlay spectra of the mixed solution of DAN and different concentrations of Nitrite; 1-8 represents blank, 10, 90, 160,240,320,400,440 ng/ml of nitrite respectively (A). Linearity curve of standard nitrite (B)

Table 2: Analytical parameters of method

Parameter	Result
Linearity range	10-440 ng/ml
Linear regression equation	y = 1.814x + 55.30
Correlation Coefficient	0.998
SD of slope	0.1882
SD of Intercept	7.9833
Limit of Detection(ng/ml)	14.4418
Limit of Quantification(ng/ml)	43.7633

With respect to Y=mX+C, X is the concentration in ng/ml, Y is the flourescence intensity

Sensitivity

The limit of detection (LOD=3.3*SD a/b, where a is the standard deviation of response and b is the slope) was found to be 14.4418 ng/ml. The limit of quantification was found to be 43.7633 ng/ml. Analytical parameters are depicted in Table 2.

Precision

The intraday precision studies were carried out through replicate analysis (n=3) of sodium nitrite corresponding to 10,90,160, 240,320,400,440 ng/ml for proposed method. The % RSD values were well within the acceptance criteria. The interday precision was also evaluated through replicate analysis (n=3) of standard sodium nitrite for five consecutive days at the same concentration levels as in intraday precision. The results of these precision assays are reported in Table 3.The developed method was found to be precise as the RSD (%) values are falling within the limit described by ICH guidelines for bioanalytical method development.

Accuracy

Accuracy of the method was assessed by recovery studies for estimation of nitrite in plasma,urine and homogenate after spiking with standard at different concentration levels of 80, 100 and 120 %. The mean recoveries are as per the table 4 at different concentration levels.

Table 3: Precision of method

Method	Concentration(ng/ml)		RSD(%)	SEM	UCL[a]	LCL[b]
Inter day	**Concentration taken**	**Mean Conc ± SD[c]**				
	10	12.93 ± 0.90	6.94	0.52	10.699	15.152
	90	88.23 ±10.04	11.37	5.79	63.299	113.158
	160	149.24 ± 3.99	6.41	2.31	139.32	159.165
	240	232.69 ± 19.61	8.43	11.32	183.985	281.399
	320	311.48 ± 37.22	11.95	21.49	219.019	403.951
	400	392.02 ± 58.56	14.94	33.81	246.55	537.49
	440	429.0371 ± 47.60	11.10	27.49	310.77	547.30
Intraday	10	12.79 ± 0.87	6.82	0.50	10.63	14.96
	90	92.19 ± 1.42	1.55	0.82	88.64	95.73
	160	163.48 ± 10.48	2.68	6.05	137.45	189.51
	240	244.59 ±1.50	0.61	0.86	240.87	248.31
	320	330.71 ± 12.00	3.63	6.93	300.88	360.54
	400	427.17 ± 3.71	0.87	2.14	417.96	436.38
	440	451.41 ±2.49	0.55	1.44	445.24	457.59

a is the Upper confidence limit at 95% of confidence, b is Lower confidence limit at 95% confidence Mean for three independent determination

Table 4: Accuracy of method

Amount of standard added (%)	Total amount of analyte found, Mean ±RSD (%)	SEM	UCL[a]	LCL[b]
80	88.06 ±1.87	0.95	83.96	92.16
100	90.16 ±1.99	1.04	85.71	94.62
120	87.60± 1.69	0.85	83.93	91.28

a is the Upper confidence limit at 95% of confidence, b is Lower confidence limit at 95% confidence

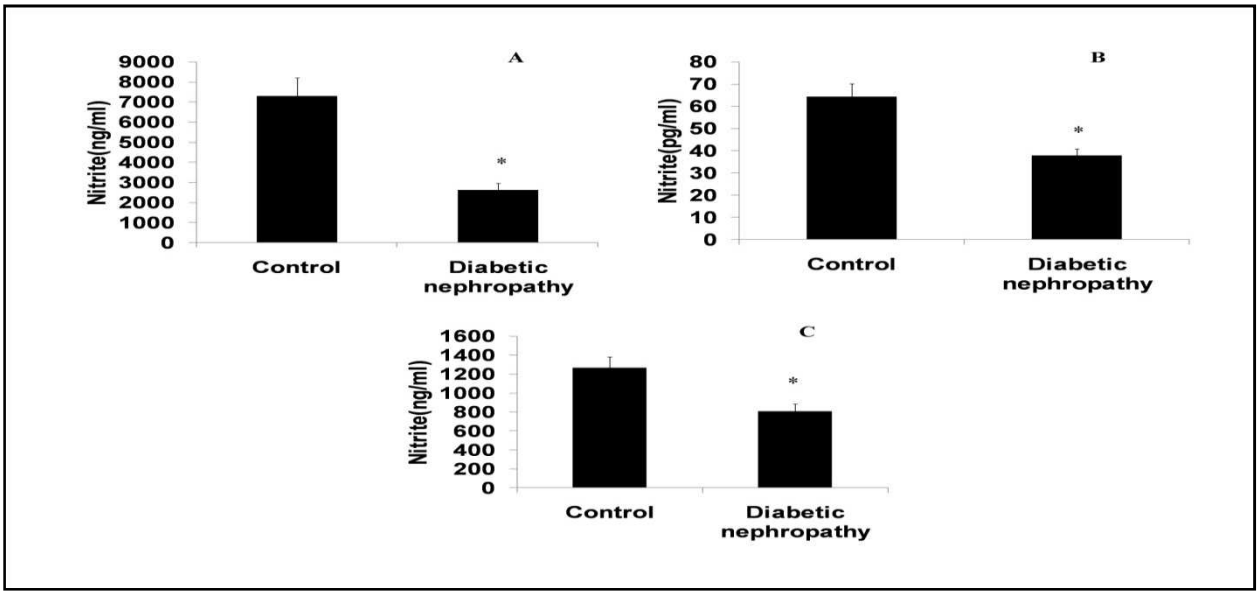

Fig. 5: Nitrite levels in plasma (A), urine (B) and kidney homogenate(C) of animals. Data are means (±SD) * $p < 0.05$ Vs. Control group; n = 6

Application of developed method

As an indicator of renal function, Blood Urea Nitrogen (BUN), creatinine clearance and urinary albumin excretion were measured. Ten weeks of diabetes adversely affected the renal function (data not shown here). Plasma NO levels in control and diabetic mice are shown in Fig. 5a. The plasma NO levels significantly decreased after 10 weeks of diabetes induction (p <0.05). The urinary and kidney homogenate NO levels were significantly reduced compared to the control group (Fig. 5b-5c, p<0.05), after 10 weeks of diabetes induction.

NO is a diffusible gas and is converted to nitrite and nitrate within seconds, thus it is difficult to directly measure changes in its concentration. Measurements of the stable end products of NO, nitrite and nitrate provide a qualitative measure of NOS activity and NO production. Since the tissue NO levels cannot be measured by the Griess method; a sensitive method is needed. Nitrite measurement in seawater medium have been reported earlier [17].The method has been also optimized for assay in brain tissue [13]. In the present studies, we measured NO levels in plasma urine and kidney tissue after 10 weeks of diabetes. Following 10 weeks of diabetes, a sharp decrease in plasma NO levels was observed Fig. 5; a similar decrease was also observed in urine and kidney tissue. The results suggest a reduction in NO in diabetic nephropathy. The results are consistent with the results obtained earlier [18]. There are several reasons accounting for diminished bioavailability in plasma. Glucose can scavenge NO, an impairment of eNOS activation, and enhanced oxidative stress quenching NO to form peroxynitrite. The formation of advanced glycation products in diabetes may also result in the consumption of endothelial NO. Another mechanism may be elevation in both asymmetric dimethyl arginine and uric acid, which can reduce endothelial NO bioavailability. NO may bind to

glycosylated deoxyhemoglobin. The reduced levels cannot be accurately measured by the Griess method.

CONCLUSION

An optimized simple spectroflourimetric method for the determination of nitrite as an indicator of NO release in different biological fluid in diabetic mouse was developed and validated over the concentration range from 10–440 ng/ml. The method has been successfully applied to estimate NO levels in diabetic nephropathy mice and satisfactory results were obtained, which demonstrates that the method is reproducible, sensitive and reliable. Owing to great pathological implications of NO in diabetes; the work may help to investigate underline mechanism of diabetic nephropathy in future.

REFERENCES

1. 1. Mathur V, Satrawala Y, Rajput MS, Vishvkarma A, Shah TD. Physiological and Pathophysiological Functions of Nitric Oxide. Der Pharmacia Lettre 2010;2(2):244-57
2. 2. Bryan NS, Grisham MB. Methods to detect nitric oxide and its metabolites in biological samples. Free Radic Biol Med 2007;43(5):645-57.
3. 3. Granger DL, Anstey NM, Miller WC, Weinberg JB. Measuring nitric oxide production in human clinical studies. Methods Enzymol 1999;301:49-61.
4. 4. Andrews AM, Jaron D, Buerk DG, Kirby PL, Barbee KA. Direct, real-time measurement of shear stress-induced nitric oxide produced from endothelial cells *in vitro*. Nitric Oxide 2010;23(4):335-42.
5. 5. Shimizu T, Onuma T, Kawamori R, Makita Y, Tomino Y. Endothelial nitric oxide synthase gene and the development of diabetic nephropathy. Diabetes Res Clin Pract 2002;58(3):179-85.
6. 6. Prabhakar SS, editor. Role of nitric oxide in diabetic nephropathy. Semin Nephrol; 2004: Elsevier.
7. 7. Erdely A, Freshour G, Maddox D, Olson J, Samsell L, Baylis C. Renal disease in rats with type 2 diabetes is associated with decreased renal nitric oxide production. Diabetologia 2004;47(10):1672-6.
8. 8. Trachtman H, Futterweit S, Pine E, Mann J, Valderrama E. Chronic diabetic nephropathy: role of inducible nitric oxide synthase. Pediatr Nephrol 2002;17(1):20-9.
9. 9. Sen U, Rodriguez WE, Tyagi N, Kumar M, Kundu S, Tyagi SC. Ciglitazone, a PPARγ agonist, ameliorates diabetic nephropathy in part through homocysteine clearance. The American Journal of Physiology- Endocinology & Metabolism 2008;295(5):E1205-E12.
10. 10. Misko TP, Schilling R, Salvemini D, Moore W, Currie M. A fluorometric assay for the measurement of nitrite in biological samples. Anal Biochem 1993;214(1):11-6.
11. 11. Kader A, Frazzini VI, Solomon RA, Trifiletti RR. Nitric oxide production during focal cerebral ischemia in rats. Stroke 1993;24(11):1709-16.
12. 12. Ohta T, Arai Y, Takitani S. Fluorometric determination of nitrite with 4-hydroxycoumarin. Anal Chem 1986;58(14):3132-5.
13. 13. Rao AM, Dogan A, Hatcher J, Dempsey R. Fluorometric assay of nitrite and nitrate in brain tissue after traumatic brain injury and cerebral ischemia. Brain Res 1998;793(1):265-70.
14. 14. Wu KK, Huan Y. Streptozotocin-Induced Diabetic Models in Mice and Rats. Current Procols in Pharmacology 2008:5.47. 1-5.. 14.
15. 15. Ames PR, Ortiz-Cadenas A, Garcia-De La Torre I, Nava A, Oregon-Miranda A, Batuca JR et al. Rosuvastatin treatment is associated with a decrease of serum oxidised low-density lipoprotein/beta2-glycoprotein I complex concentration in type 2 diabetes. British Journal of Diabetes Vascular Disease 2010;10(6):292-9.
16. 16. Krafte-Jacobs B, Brilli R, Szabo C, Denenberg A, Moore L, Salzman AL. Circulating methemoglobin and nitrite/nitrate concentrations as indicators of nitric oxide overproduction in critically ill children with septic shock. Crit Care Med 1997;25(9):1588-93.
17. 17. Liu C, Zhao M, Ren C, Yang G, Li P, Han Y. Direct measurement of nitric oxide in seawater medium by fluorometric method. Chinese Journal of Analytical Chemistry 2009;37(10):1463-7.
18. 18. Prabhakar S, Starnes J, Shi S, Lonis B, Tran R. Diabetic Nephropathy Is Associated with Oxidative Stress and Decreased Renal Nitric Oxide Production. J Am Soc Nephrol 2007;18(11):2945-52. doi:10.1681/asn.2006080895.