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Effect of high dose thiamine on the levels of urinary protein biomarkers in diabetes mellitus type 2

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ABSTRACT

The proteomics is known to be a valuable field of study and has become one of the most attractive sub-disciplines in clinical proteomics for human diseases. In the present research work, the levels of urinary protein biomarkers of diabetes mellitus type 2 using proteomic technology have been identified and characterized. Effect of high dose thiamine has also been observed on the levels of these marker proteins. Above 100 type 2 diabetic patients, and 50 same age and sex-matched normal healthy controls were recruited from the Sheikh Zayed Hospital, Lahore, Pakistan and 40 diabetic and 20 control have completed the trial. The urine samples from control and diabetic groups before or after thiamine therapy were further analyzed and identified by 2-D liquid chromatographic system (HPLC) and mass spectrometry MALDI-TOF/TOF and microTOF analysis. All the samples belonging to the control and diabetic groups were then analyzed by ELISA and estimated the levels of some proteins which were found to vary. In the urine samples, the levels of transthyretin, AMBP, haptoglobin precursor were found to decrease while albumin, zinc α 2 glycoprotein, RBP4 and E cadherin were found to increase in the diabetic patients as compared to the controls. The level of albumin in the urine samples of diabetic patients only decreased by 34% after thiamine therapy as compared to the controls and the placebo, while other urinary protein markers did not show a significant change after the therapy. Assessment of the levels of these biomarkers will be helpful in the diagnosis and treatment of diabetes mellitus type 2.

1. Introduction

Diabetes mellitus is one of the most widely occurring human ailments. Diabetes is increasing in incidence in the South Asian countries and elsewhere to epidemic proportions. It is the 2nd most common disease in industrialized countries and is projected to become one of the world's main disablers and killers within next few years. Type 2 diabetes mellitus is the most prevalent form of the syndrome and diabetic nephropathy is a well-known complication of type 2 diabetes with renal failure and is considered the cause of death in most of cases. Diabetes patients with renal disease have a poor prognosis [1]. Proteomics has become one of the most important research topics and has recently become available for large-scale protein analysis. These technologies are now being applied to diabetic nephropathy to biomarker discovery in several diseases with the hope of finding novel biomarkers for earliest diagnosis of the diseases at their very beginning phase, and for prediction of therapeutic response, survival and recurrence. Searching for novel biomarkers can be done using tissues and/or biofluids. The urine is an ideal biofluid for biomarker discovery in kidney diseases and diabetes mellitus. Urine samples obtained from patients with other diseases or disorders that have clinical, biochemical and metabolic profiles similar to those of the disease of interest must be included as the other controls. Finally, a single ideal biomarker may not exist for each disease. Therefore, evaluating a panel of multiple biomarkers may be required [2].

Quantitative and qualitative analyses of urinary proteins have been used to study renal physiology and as diagnostic tool in renal and systemic diseases. Plasma retinol binding protein-4 and its concentrations were elevated in human subjects with impaired glucose tolerance and type 2 diabetes. Both albumin and retinol-binding protein (RBP) were also reported to be associated with retinopathy [3]. Proteins of human urine and their concentrations were analyzed by two-dimensional electrophoresis [4]. Characterization of diabetic nephropathy by urinary proteomics was analyzed and identification of a processed ubiquitin form as a differentially excreted protein in diabetic nephropathy patients was studied [5]. Identification of urinary soluble E-cadherin was done as a novel biomarker for diabetic nephropathy [6]. Haptoglobins are a group of serum proteins, originally identified and diagnostic values were

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determined [7]. Determination and quantification of zinc-alpha-2-glycoprotein as a potential serum biomarker for prostate cancer were observed in patients with metabolic syndrome by ELISA and LC–MS/MS [8]. Measurement of levels of protein human α_1-microglobulin (α_1m) was studied in the sera and urine of patients with various liver diseases [9]. Transthyretin and its miracle function and pathogenesis were observed [10]. In addition to being an early marker of diabetic nephropathy, microalbuminuria is also known to be a predictor of coronary heart disease and peripheral vascular disease, as well as increased risk of mortality in patients with type 2 diabetes.

Thiamine (vitamin B_1), deficiency results in a disease called beri beri, in which cardiovascular, neurological and dermatological complications arise. It has a known history of use for alcoholic neuropathy and has been shown to be helpful in alleviating alcoholic cirrhosis. Animal studies with high dose thiamine therapy have shown to reduce diabetic nephropathy (microalbuminuria) and lipid disturbances. High prevalence of low plasma thiamine concentration in diabetes was linked to marker of vascular disease. Recently, high dose thiamine therapy has been shown to reverse the condition of early stage diabetic nephropathy in diabetes type 2 patients [11]. This study reports identification of some of the protein biomarkers in diabetes mellitus type 2 in the local population and the effect of high dose thiamine therapy on the level of these biomarkers.

2. Experimental

2.1. Sample collection

125 diabetic and 50 controls individuals (age 35–65 years, equal number of male and female) were initially enrolled in this study using the stringent inclusion and exclusion criteria recommended by the American Diabetes Association for type 2 diabetes between October 01, 2006 and December 01, 2006. Only 40 diabetic patients and 20 controls (aged 35–65 years, equal number of men and women) completed the trial period over six months and others were dropped out due to some reasons. Half the number of patients were administered 3 × 100 mg tablets/day for three months, while the other half were treated as placebo. The treatment period was followed further by a 2 month washout period. A detailed individual patient's present and past family, occupational, socio-economical history was recorded in this study after consent of each patient.

2.2. Ethical approval of the study

Ethical approval for the study was taken by the Ethical/Protocol/Synopsis Committee of FPGMI (Sheikh Zayed Federal Post Graduate Medical Institute, Lahore, Pakistan). The study was assigned the number as Eth/P 609/FPGMI 2006. It is internationally registered and clinical trial number assigned was CTRI/2008/091/000112, with the identification number CTRI/2008/091/000112 with the World Health Organization's (WHO) international clinical trials registry platform search portal (http://www.ctri.in/Clinicaltrials/ViewTrial.jsp?trialno=203trialid=CTRI/2008/091/000112apps.who.int/trialsearch/trial.aspx?trialid=CTRI/2008/091/000112).

2.3. Estimation of biochemical parameters

Blood and 24 h urine samples were collected from the selected subjects. All subjects (control and diabetic individuals) provided urine containers to store urine samples in the early morning after a 24 h overnight fast. The urine samples were immediately frozen and stored at −80 °C to prevent protein degradation. Relevant biochemical parameters were determined as described previously [12]. Total urinary proteins were determined by Bradford assay [13].

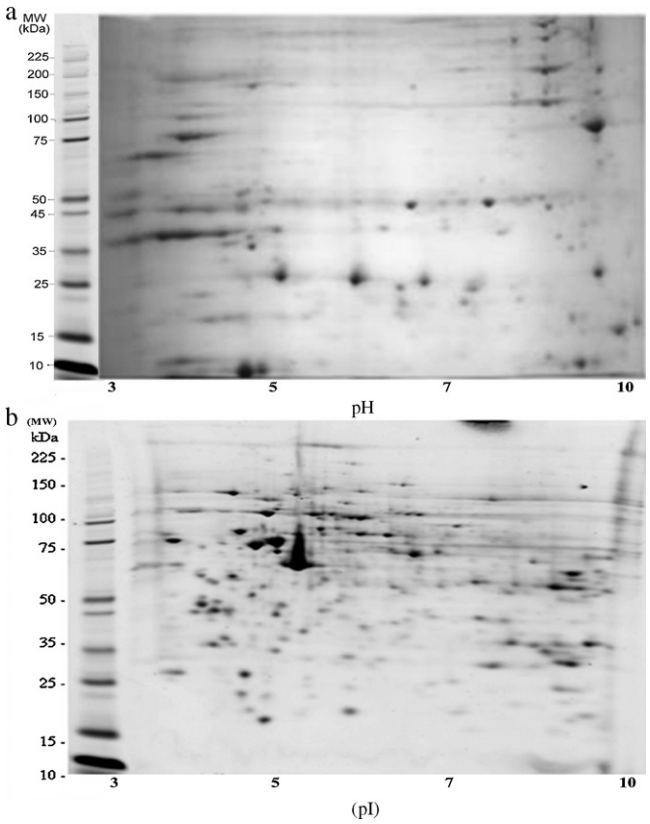

Fig. 1. 2-D gel analysis of human urine proteins of representative (a) samples of normal with comparison of (b) diabetic groups.

2.4. Sample preparation for proteomic analysis

Urine samples (1 ml) were collected in 1.5 ml of eppendorf vial. The samples were thawed, mixed and passed through 0.34 mm Whatman chromatography paper (Whatman, Maidstone, UK). Urine samples were centrifuged at 3000 rpm for 10 min at 4 °C to remove debris. After removal of cell debris and nuclei, the supernatants were fractionated by 1% TFA precipitation for 10 min followed by centrifugation at 10,000 rpm for 5 min at 4 °C. The resultant pellet was resuspended in 100 μl HPLC water. Protein concentration of each sample was measured by spectrophotometry using a Biorad protein microassay based on the method of Bradford. 2-D gel electrophoresis and image analysis were performed using the recommended protocols according to the referred method as shown in Fig. 1 [14].

2.5. 1st dimension analysis: chromatofocusing (CF)

Modern proteomic techniques such as two D liquid chromatography (2D-LC) and reverse phase high performance liquid chromatography (RP-HPLC) coupled with mass spectrometry (MS) were used to identify and characterize the urinary protein biomarkers in diabetes type 2. Equilibration of 100 μl of urine sample was done using chromatofocusing (CF) start buffer up to a final volume of 2 ml containing 5 mg of total urinary proteins. HPCF 1D column (2.1 mm × 250 mm, GE Healthcare, USA) was used where mobile phase comprised start buffer pH 8.5 ± 0.1, eluent buffer pH 4.0 ± 0.1, high ionic strength wash and HPLC grade water with flow rate of 0.2 ml/min at ambient temperature. Detection was done at

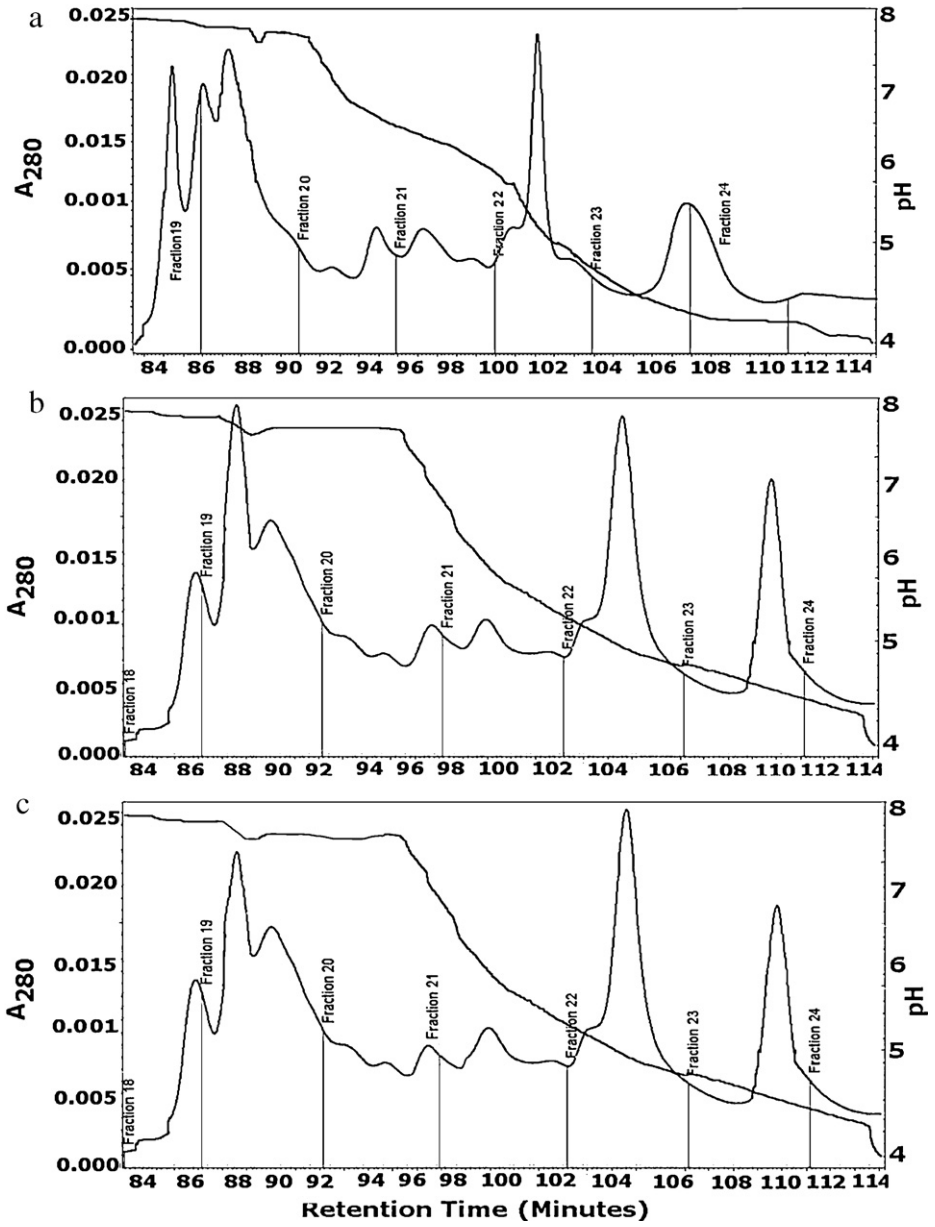

Fig. 2. Analysis of human urine proteins of representative (a) samples of normal with comparison of (b) diabetic groups before thiamine therapy, (c) diabetic group after thiamine therapy by first dimension chromatography (chromatofocusing).

UV 280 nm in the ProteomeLab PF2D (Beckman Coulter Inc., USA) using 600–1000 psi back pressure. The fractions at 0.3 pH intervals were collected in a 96 deep-well plate [15].

2.6. 2nd dimension analysis: reverse-phase high performance liquid chromatography

Selected fractions from the CF column were analyzed in second dimension reverse phase high performance liquid chromatography (RP-HPLC) using a column (Phenomenex Luna C18 2 mm × 100 mm) with 2.5 μM particles size (Phenomenex) with mobile phase as solvent A: 5 mM ammonium acetate in 0.1% formic acid, solvent B: acetonitrile with standards: Ehrenstorfer Mix 34 (Agilent 1100, UK). HPLC: Agilent 1100 binary gradient HPLC system was used equipped with well-plate autosampler, column oven and DAD detector (Agilent) with run time 19 min, post run time 5 min, flow rate 0.30 ml/min, injection volume 10 μl column temperature 40 °C. Detection was done at UV 214 nm using 1500–2000 psi back pressure [16].

2.7. Mass spectrometric (MALDI-TOF) analysis and database searching

The fractions showing peaks of interest were selected from RP-HPLC and 1 M NH_4HCO_3 and 10 mM DTT were added to each fraction from HPLC column to final concentrations of 100 and 1 mM, respectively. The resulting fractions were incubated at 60 °C for 10 min and then 1 μl was subjected for intact mass analysis to mass spectrometry. Subsequent protein identification for intact protein was carried out using matrix-assisted laser desorption/ionization time of flight tandem mass spectrometry (MALDI-TOF-MS) (ABI 4700 Proteomic Analyzer, Applied Biosystems Company, USA) on a reflective setting. Then the strongest seven peaks were selected to obtain tandem mass spectrometry (MS/MS) data acquired when the mass-to-charge ratio (m/z) was above 8000–95,000 Da [17].

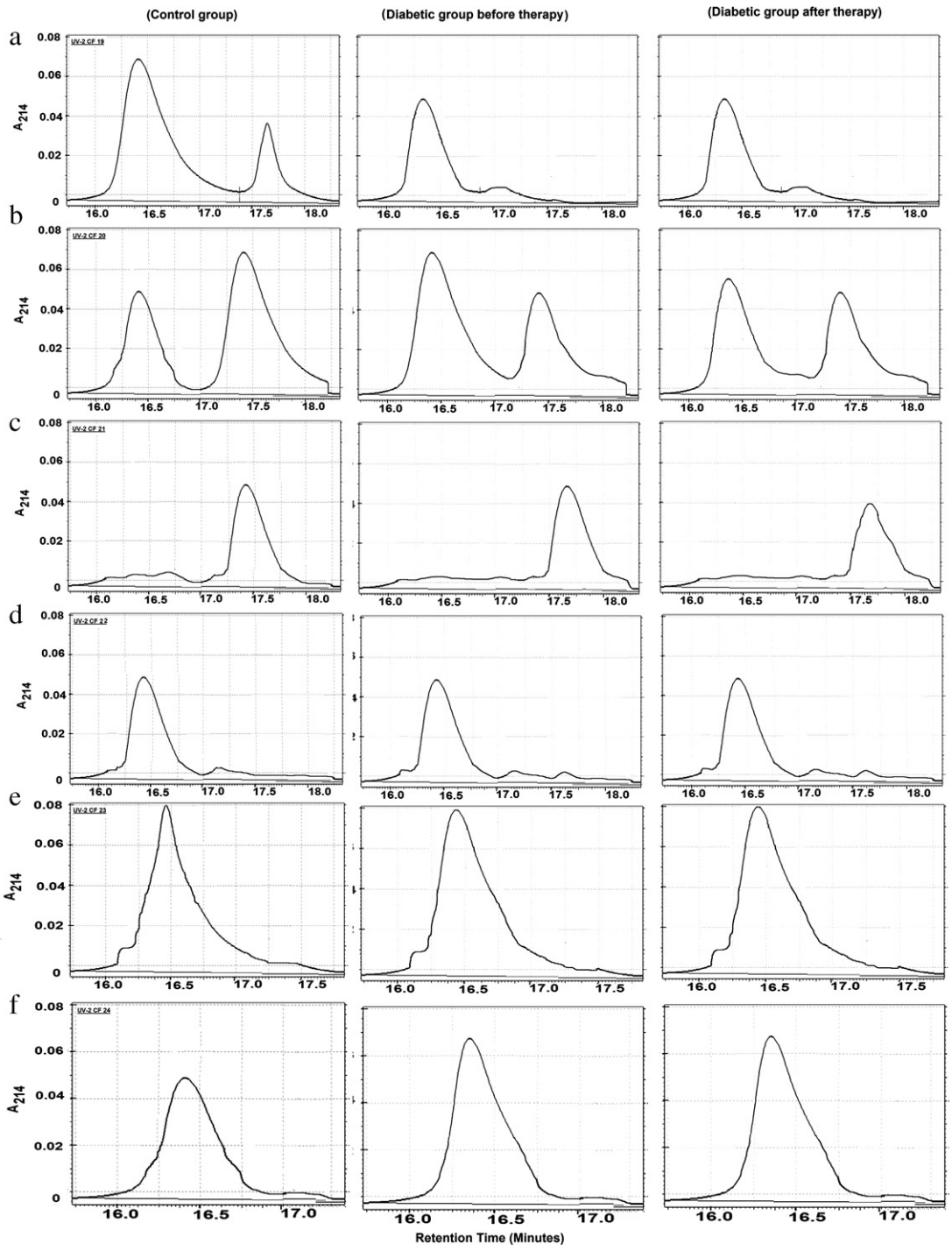

Fig. 3. Second dimension RP-HPLC analysis of comparison of normal control human urine with diabetic groups before and after therapy from fractions 19 (a), 20 (b), 21 (c), 22 (d), 23 (e) and 24 (f) obtained from the first dimension analysis.

The remaining fractions after incubation were subjected to enzymatic digestion with trypsin (Promega, Madison, WI, USA) as above 1 ml of trypsin solution (100 ng/ml) in 50 mM CH_3COOH was added and digestion was done by incubation in a shaker at a medium speed at 37 °C for 24 h. The tryptic digests were evaporated and then reconstituted in 3 ml HPLC-grade water and purified with a ZipTipc18 (Millipore, Billerica, MA, USA) using the procedure recommended by the manufacturer. The purified peptides were eluted from the ZipTip directly onto a stainless steel MALDI target plate (Bruker Daltonics, Bremen, Germany) with 1 μl of a saturated solution of α-cyanohydroxy sinapinic acid in a mixture 0.1% TFA:ACN (2:1, v/v). The peptide mixture after drying at room temperature was analyzed with a Reflex IV MS: microTOF (Bruker Daltonics, Germany). MS: microTOF (Bruker Daltonics) time of-flight mass spectrometer (Bruker Daltonics, Bremen, Germany) [18].

2.8. Protein analysis by ELISA

Quantification of the proteins, which were found to vary between the controls and the diabetics, was done in all the diabetes mellitus type 2 patients and the controls by ELISA methods. Levels of haptoglobin precursor, albumin, α-1-microglobulin/bikunin

Table 1
Identification of protein biomarkers in human urine of diabetes type 2 by 2D-liquid chromatography and mass spectrometric analysis.

S. no.	CF[a]/pI[b]	Protein name	Accession no.[c]	Actual protein MW[d] (Da[e])/pI	Observed protein MW (Da)	Methods[f]	Peptide matches	MOWSE[g] score	Sequence coverage (%)
1	6.38–6.08	Haptoglobin precursor	P00738	45,206/6.13	45,860.8	PMS, MS	37/37/61	91	50.7
2	6.08–5.78	Albumin	P02768	71,658.1/5.9	71,317.2	PMS, MS	63/60/46	278	52
3	6.08–5.78	AMBP[h]	P02760	38,999/5.95	39,886.3	PMS, MS	30/30/50	105	50
4	5.78–5.48	RBP4[i]	P02753	23,010/5.76	23,371.4	PMS, MS	28/25/51	169	50.2
5	5.48–5.18	Transthyretin	P02766	15,877/5.5	15,991.1	PMS, MS	15/15/58	442	76.2
6	5.18–4.88	Zα 2G[j]	P25311	33,872/5.6	34,079.1	PMS, MS	61/57/81	153	71.5
7	4.88–4.58	E cadherin	P12830	97,457/4.6	97,853.0	PMS, MS	67/67/86	107	51.6

[a] CF, chromatofocusing.
[b] pI, isoelectric point.
[c] Accession number in National Center for Biotechnology Information (NCBInr) databases.
[d] MW, molecular weight.
[e] Da, dalton.
[f] PMF, MS (peptide mass fingerprinting/matrix-assisted laser desorption ionization, time of flight, mass spectrometry [MALDI-TOF/MS]).
[g] MOWSE (MOWSE score is used in peptide mass fingerprinting as it is similarity score derived from statistical model of probability of matching peaks).
[h] AMBP, α-1-microglobulin/bikunin precursor.
[i] RBP-4, retinol binding protein-4.
[j] Zα 2G, zinc α 2 glycoprotein.

precursor (AMBP), retinol binding protein 4 (RBP4), transthyretin, zinc α 2 glycoprotein (Zα 2G) and E cadherin were determined. All the assays were based on non-competitive sandwich ELISA methods using commercially available kits and protocols and the assays were performed according to the manufacturer's specifications as described in the kits. Haptoglobin precursor and zinc α 2 glycoprotein (Zα 2G) were estimated by the kits provided by Biovendor Research and Diagnostic Products Company, Candler, NC, USA, while albumin was determined by kit supplied from Alpha Diagnostic International, TX, USA. The α-1-microglobulin/bikunin precursor (AMBP), retinol binding protein 4 (RBP4) and transthyretin were assayed by kits manufactured by Uscn Life Sciences, Inc. Wuhan, China and E cadherin was assayed by the kit provided by Genway Bio Company, Diago, CA. All the assays were measured spectrophotometrically at a wavelength of 450 ± 2 nm. The concentrations of proteins in the samples were then determined by comparing the O.D. of the samples to the standard curve.

2.9. Statistical analysis and softwares used

Statistical methods: all data were collected and analyzed by SPSS software 17.0 (SPSS, Chicago, IL, USA). Values were expressed as means ± SD. The difference among groups was compared with single-factor ANOVA. Pearson correlation and step-wise regression analysis were used to explore the relationship between urinary proteins and the clinical characteristics of subjects. All tests were two tailed and $p < 0.05$ was considered significant. For protein profiling 32Karat, Hystar3.1 Galaxies version 1.7, Compass3.3 and MASCOT softwares were used for identification and characterization of the biomarker proteins in all the samples.

3. Results and discussion

For biomarker discovery, although a large number of biomarker candidates have been identified, they are neither validated in a large cohort nor ready for routine clinical practice at present. Similarly, a number of new therapeutic targets have been identified, but no new drugs or therapies have been developed based on such therapeutic targets identified by the proteomic approach [4]. This study reports identification of some of the protein biomarkers in diabetes mellitus type 2 in the local population and focused mainly to biomarker discovery, some of these studies also revealed potential new therapeutic targets.

In this study, the most advance protocols of proteomics study and technology such as chromatofocusing (CF) and RP-HPLC coupled with mass spectrometry (MS) were used. In the present research work, the urine samples were initially analyzed by 2-D liquid chromatography in which the samples were first analyzed by chromatofocusing and the selected fractions from the first analysis were further analyzed by reverse phase chromatography. The identity of selected fractions was established by analysis of the intact protein and their tryptic digest by mass spectroscopy. Thereafter the levels of the proteins which were varied in diabetic samples or the samples from diabetic patients were determined by ELISA assay methods.

3.1. 2-D liquid chromatography analysis (1st dimension: chromatofocusing)

The 1-D analysis of urine of control and diabetic samples elution profile of the proteins in the various fractions as analysis by the chromatographic columns has been shown (Fig. 2). The protein composition in the fraction range 19–24 seemed to vary between that of healthy control and the samples from the diabetic patients. The expression of some of protein levels in the fractions 20, 22, 23 and 24 was quite low in the healthy samples whereas in the diabetic samples the significant levels of at least three proteins in these fractions (Fig. 2a). Similarly, the expression of some of proteins in the fractions 19, 20 and 21 was down regulated in the diabetic urine samples (Fig. 2b). The fractions from 19 to 24 as obtained from the first dimension analysis were analyzed further by reverse phase chromatography column in the representative samples of all groups of individuals. The 1st dimension chromatogram analysis after the effect of high dose thiamine on the diabetic urine sample has been shown in Fig. 2c. There were some significant changes observed in the pattern of protein profile in some of the peak areas in the diabetic group after thiamine therapy after washout period. The selected fractions showing the variations have been selected for the further second dimensional analysis.

3.2. 2nd dimension analysis (RP-HPLC)

The second dimension (RP-HPLC) analysis of all the samples of control and diabetic before and after thiamine therapy has been performed and chromatogram of the one representative sample from the control and diabetic individuals has been shown in Fig. 3. The elution profile after the RP-HPLC analysis from the different

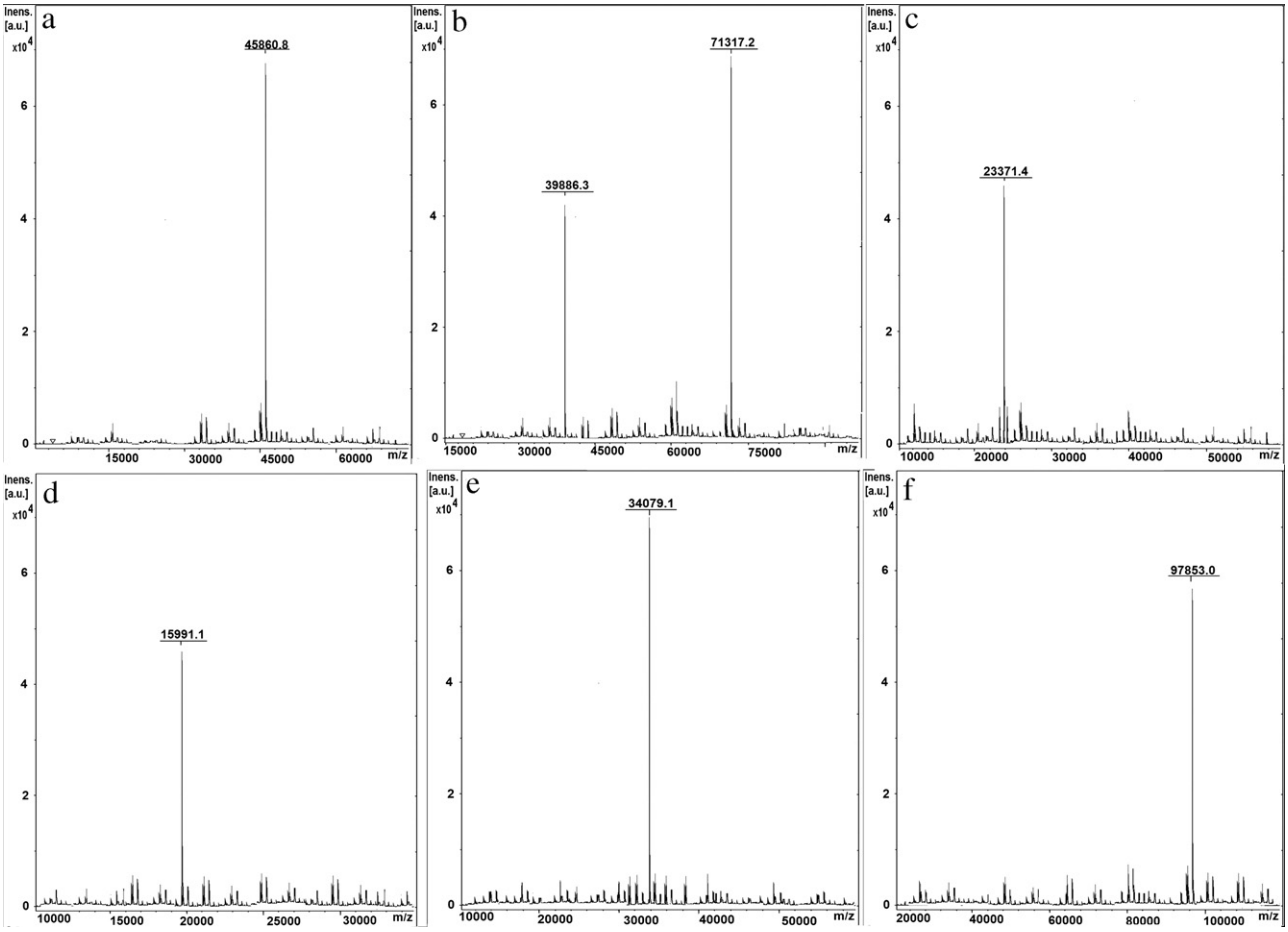

Fig. 4. Mass spectrometric analysis (MALDI-TOF) of four protein species showing their intact masses, one from the fraction 19 (a) and two from fraction 20 (b) and one each from fractions 21 to 24 (c–f).

fractions of CF as 19–24 from the control and diabetic group has been obtained because these CF fractions showed the variations in the peak areas of protein profiles of both groups. The analysis of the control group showed that there were two proteins expressed in fractions 19 and 20 (Fig. 3a and b) and one prominent protein peak in the other fractions 21–24 of control group from 2nd dimension analysis as shown in Fig. 3c–f. The analysis of the diabetic group showed that there was one prominent protein expressed in fraction 19 as compared to two protein peaks in case of control sample after RP-HPLC dimension analysis. The CF no. 20 of the diabetic urine sample showed two prominent peaks but with different levels of expression as compared to the control sample. Also, fractions 21–24 of the diabetic group showed significantly higher expression level of only one of the significant proteins in the diabetic group. This type of pattern of protein composition in these corresponding fractions seemed very similar in the control group. The expression of the protein pattern and composition of these selected fractions were also very similar in the diabetic group at the end of 3 months therapy and further two months of washout period after treatment except on some of the protein peak areas has been decreased after therapy in some of the fractions like CF 19. The proteins fractions from 19 to 24 after the RP-HPLC column contain proteins which were in the pI range of 6.38–6.08, 6.08–5.78, 5.78–5.48, 5.48–5.18, 5.18–4.88 and 4.88–4.58, respectively (Table 1) were analyzed by the Compass3.3 and Hystar3.1 HPLC softwares and are summarized in Table 1.

3.3. Mass spectrometric analysis (MALDI-TOF)

The six protein fractions which were varied and obtained from the reverse phase chromatography in the representative sample of various groups were analyzed by mass spectrometric analysis in order to confirm their identity. The intact molecular mass of each of these proteins as well as the mass profile of tryptic digest of these proteins was also determined. Fig. 4a shows the analysis of the protein present in CF 19 in the control and diabetic sample which showed the protein mass of 45,860.8 Da which corresponding to haptoglobin precursor (pre-albumin protein). Fig. 4b shows the analysis of CF 20 of the two proteins taken together in the same sample which showed the protein mass of the two species 39,886.3 Da and 71,317.2 Da which corresponds to α-1-microglobulin/bikunin precursor (AMBP) and albumin, respectively. Similarly Fig. 4c shows that mass of the protein present in fraction 21 of 1st dimension chromatography and purified by 2nd dimension chromatography was found to be 23,371.4 Da, which was the same as that of retinol binding protein 4 (RBP4). Similarly the protein species in fraction 22 of 1st dimension analysis and purified by reverse phase column in the intact state as shown in Fig. 4d was found to be transthyretin precursor with molecular weight of 15,991.1 Da. The CF no. 23 showed that mass of the protein present which was found to be 34,079.1 Da and corresponding to that of zinc α 2 glycoprotein (Zα 2G) as shown in Fig. 4e. Similarly the protein species in fraction 24 after the 1st and 2nd dimension analysis

Table 2
Quantification of protein biomarkers by ELISA in urine samples of control, diabetic placebo and diabetic thiamine-treated groups.

S. no.	Variable[A]	Placebo group[B]		Thiamine treatment group[C]		% Age change
		Baseline	At 5 months	Baseline	At 5 months	
1	Haptoglobin precursor (μg/ml)	13.82 ± 2.35	13.80 ± 2.35	13.84 ± 2.35[a]	14.20 ± 2.25[a]	+2.9
2	Albumin (μg/ml)	56.8 ± 19.4	54.5 ± 18.45[a]	56.9 ± 19.45[a]	37.0 ± 11.54[b]	−34.9
3	AMBP (ng/ml)	27.8 ± 8.35	26.5 ± 8.35	27.8 ± 8.35[a]	26.5 ± 8.35[a]	−4.6
4	RBP4 (ng/ml)	7.9 ± 1.79	7.9 ± 1.79[a]	7.8 ± 1.79	7.9 ± 1.79[a]	+1.28
5	Transthyretin (ng/ml)	12.7 ± 2.35	16.5 ± 2.35[a]	12.8 ± 2.35[a]	16.5 ± 2.95[a]	−28.9
6	Zinc α 2 glycoprotein (μg/ml)	67.4 ± 28.35	67.35 ± 28.35	67.35 ± 28.35[a]	68.25 ± 28.35[a]	+1.34
7	E cadherin (μg/ml)	4.09 ± 1.67	4.08 ± 1.67[a]	4.08 ± 1.67[a]	4.03 ± 1.67[a]	−0.9

[a] $p > 0.05$ (statistically non significant).
[b] $p < 0.001$ (statistically highly significant) comparison of control with placebo and thiamine treated groups at 3 months therapy and 5 months washout period.
[A] Data are mean ± SD.
[B] Figures are the average of 20 samples.
[C] Figures are the average of 40 samples.

and purified in the intact state as shown in Fig. 4f was found to be E-cadherin with the molecular weight of 97,853.0 Da. After the CF and MS analysis as clearly shown in Table 1, the sequence coverage of all the identified proteins were above the 50%. Table 1 also shows the PI values with an accession number of identified protein biomarkers showing the molecular weight, peptide matches and MOWSE score after peptide mass fingerprinting MASCOT software analysis.

The analysis of peptide profile for each of the protein as done by MASCOT software search engine, confirmed the identity of all these proteins mentioned above. On the basis of this data, the total seven proteins identified in the six fractions were haptoglobin precursor (pre-albumin protein), α-1-microglobulin/bikunin precursor (AMBP), albumin, retinol binding protein 4 (RBP4), transthyretin precursor, zinc α 2 glycoprotein (Zα 2G) and E-cadherin, respectively (Fig. 4).

3.4. Quantification analysis by ELISA

In the urine samples, the levels of transthyretin, AMBP, haptoglobin precursor were found to be lower by 30.8, 55.2, and 81.45%, while albumin, zinc α 2 glycoprotein, RBP4 and E cadherin were found to be higher by 486.5, 29.23, 100 and 693%, respectively in the diabetic patients as compared to the controls as described previously [28]. The effect of high dose thiamine on the levels of these urinary protein biomarkers has also been observed in all the placebo and thiamine treated groups at baseline, three month therapy and washout periods. There were 2.9%, 1.28% and 1.34% increase non significantly after thiamine therapy in the haptoglobin precursor protein, RPB4 and zinc α 2 glycoprotein, respectively in the diabetic thiamine treated groups. These changes were non significant and in placebo group similar changes were seen (Table 2). While in the proteins like albumin, AMPB, transthyretin and E-cadherin showed 34.9%, 4.6%, 28.9% and 0.9% decrease after thiamine therapy (at 3 months and further two months washout period) in the diabetic group receiving thiamine treatment as compared with placebo group. The level of albumin in the urine samples of diabetic patients only decreased significantly by 34.9% after thiamine therapy as compared to the controls and the placebo while other urinary protein markers did not show a significant change after the therapy as shown in Table 2.

Determination of protein composition of normal urine may lead to an increased understanding of renal physiology and will build a database for comparison to urine from patients with various renal diseases. During the past 5 years, urinary proteomics has been extensively applied to biomedical research with ultimate goals for biomarker discovery, diagnostics and prognostics for the nephropathy in diabetes. Urinary proteomics has been extensively applied to biomarker discovery, diagnostics and prognostics for several kidney and non-kidney diseases during the past 5 years. Using various kinds of proteomic techniques, especially 2-DE, 2-D DIGE, SELDI-TOF MS, CE–MS and microfluidic technology on a chip, several urinary biomarker candidates have been identified and characterized. Some of these biomarker candidates have been already validated in a blinded independent set of urine samples, while a few of them have been confirmed in the affected tissues. A number of studies have clearly shown that urinary proteomics can be used not only for diagnostics but also for prognostics of kidney and non-kidney diseases [19].

In recent era, 2-D liquid chromatography based methods were used and has been most successful in identifying the protein biomarkers specific to various diseases. Identification and characterization of protein biomarkers for different diseases using the 2-D LC and followed by mass spectrometry MS/MS helped us to identify some of the levels of proteins expressed as up or down regulated in the diabetes type 2 as compared with normal healthy control samples. In the present research work, a novel 2D-LC/TOF-MS approach to comprehensive serum and urine screening was used to provide for accurate and sensitive searching of protein biomarker in serum and urine samples of diabetic patients and normal individuals [20]. This modern technology has been used for the searching of biomarker which is specific to the type 2 DM in local Pakistani population and this has been demonstrated to help in the monitoring and control of this disease. Butt and Riaz have studied protein profiling of human urine in diabetic hypertensive nephropathy versus normal healthy controls in Pakistani diabetic population [21].

Thongboonkerd [19] has analyzed some of the proteins present in the kidney of type 1 DM model of murine species. He has found differential expression of some of the proteins by using the capillary electrophoresis coupled with the mass spectrometry in the urine samples from both types of DM as 1 and 2 in the diabetic patients having micro/macroalbuminuria or not. He has identified some of high abundance urine proteins and peptides like albumin which was differentially expressed in the two groups. Moreover, the expression of protein profiles from the urine samples was also compared from the diabetic patients having nephropathy with the normal healthy control individuals by using 2-D gel electrophoresis followed by mass spectrometry MALDI-TOF.

To date, microalbuminuria is the best available non-invasive marker for the detection of diabetic nephropathy. In one research study, the scientists utilized a similar strategy or urinary proteome profiling of type 2 diabetes patients with normoalbuminuria, microalbuminuria, and overt proteinuria compared to control subjects. 2-D DIGE analysis revealed significantly differing levels of several proteins among groups. They adopted a 2-D DIGE to evaluate the urinary proteome profile of the patients with long-standing diabetes, impaired renal function and overt proteinuria. The results showed that urinary excretion levels of many proteins were signif-

icantly increased, while some of the proteins were decreased in the urine of diabetic patients as compared to healthy controls [22]. Similar results were obtained in our study as level of four proteins were elevated in the diabetic individuals having type 2 diabetes and three proteins were down regulated as compared to control one (Table 2 and Figs. 1–3).

Dihazi et al. [5] have found the increased urinary excretion of albumin is a potential early indicator both for glomerular injury and for systemic endothelial injury in chronic disease processes such as diabetes and atherosclerosis and in acute illness or surgery. Consequently, there has been interest in examining whether urinary albumin excretion can serve as a marker for kidney diseases such as diabetes. Urine total NAG excretion was also higher in patients with both macro- and microvascular complications [23]. In one study, patients with associated hypertension had higher urinary levels of albumin and b2-microglobulin, regardless of whether complications were present or not. In our study, we also found an increase of 486% of albumin in the diabetic samples that showed the presence of diabetic nephropathy with high proteinuria. In addition to albumin, tubular proteins, namely, RBP and b2-microglobulin, also showed increased excretion in patients with complications. RBP excretion was, however, markedly higher only in patients with microvascular complications [23]. Elevated level of RBP4 in the humans showing insulin resistant in the obesity and type-2 diabetes has induced the expression of some enzymes involved in the gluconeogenesis like phosphoenolpyruvate carboxykinase in hepatic cells. It also helps in impairment of the signaling in insulin in the muscle tissues. It may be seen that low levels of RBP-4 in the case of nephropathy in diabetes may rise up the sensitivity of insulin which produce spontaneous hypoglycemia [24].

Zinc-α 2-glycoprotein was the urinary protein that is second most in its abundance in the case of diabetic nephropathy. Research work has showed and identified that zinc-α 2-glycoprotein with three other major proteins like α 1-acid glycoprotein, α 1-microglobulin, and IgG in the type 2 diabetes were the specific biomarkers for the diabetic nephropathy [25]. We also observed the elevated level of RBP4 and zinc-α 2-glycoprotein in or population having type-2 diabetes. ELISA of urine samples from an independent group of diabetic patients confirmed a marked increase of urinary albumin, RBP4, Zα 2G and E cadherin in the diabetic patients of local population.

Altland and co-workers have observed that transthyretin is also known as pre-albumin and is a protein binding with the thyroid hormone. It transports a little portion of thyroxine secretion from the bloodstream to the tissues and cells of brain. It has also been reported as a specific protein biomarker which is suitable for the assessment of nutrition in the patients with acute or chronic renal failure [26]. Fernández et al. [9] have studied that the AMBP contains both α 1-microglobuin and light chain of inter α trypsin inhibitor such as bikunin. Bikunin is a vital substance which is anti inflammatory and used to modulate the processes such as inflammatory and low levels of this protein may affect immunocompetence in the renal and diabetic diseases. The other part of AMBP protein is α1-microglobulin which is present in the urine and linked with the process of albuminuria in some of the world population like in the Chinese, Asian and Indians having type 2DM. In similar study, urinary α 1-microglobulin also showed the proximal tubular dysfunction and used as specific protein biomarker for the early diagnosis of diabetic nephropathy and renal diseases [27]. Our results of ELISA of urine samples from the normal healthy controls also confirmed a marked increase of urinary haptoglobin precursor, AMBP protein and transthyretin in the Pakistani population.

In the present research work and current study, we have also used a modern technique like 2-D LC followed by mass spectrometry MS/MS TOF to identify and characterize some of protein biomarkers in the human urine and that exhibited the up or down regulation in the expression of these proteins in the diabetes type 2 as compared to normal healthy control. Seven potential up and down regulated urinary biomarkers for diabetes type 2 found in this study were haptoglobin precursor (pre-albumin protein), α-1-microglobulin/bikunin precursor (AMBP), albumin, retinol binding protein 4 (RBP4), transthyretin precursor, zinc α 2 glycoprotein (Zα 2G) and E-cadherin. The identification and characterization of these protein biomarkers in diabetes type 2 showed an important step forward in advancing not only understanding but also in the treatment for this disease.

The discovery of these marker proteins might thus provide a help in detection and treatment for this silent killer disease known as diabetes. Variation in the levels of these identified protein biomarkers has been reported in other pathological states. Assessment of the levels of these biomarkers will be helpful in diagnosis and cure of diabetes mellitus type 2.

4. Conclusion

In the present research work, modern advance technologies such as proteomics and mass spectrometry analysis in the diabetic and normal healthy individuals were applied. In the urine samples of diabetic group, the increased levels of albumin, retinol binding protein 4 (RBP4), zinc α 2 glycoprotein (Zα 2G) and E-cadherin as compared to the control samples were observed. The level of haptoglobin precursor (pre-albumin protein), α-1-microglobulin/bikunin precursor (AMBP) and transthyretin precursor was downregulated in the diabetes and elevated in the control subjects. Elevated levels of albumin, RBP4, Zα 2G and E-cadherin in diabetes were found to be a powerful independent risk determinant. The effect of high dose thiamine therapy on the levels of these urinary protein biomarkers has also been observed. Our data further consolidate the evidence that these above mentioned proteins act as urinary biomarkers for diabetes mellitus type 2.

The next step of proteomics will move onward to verification of these biomarkers in a much larger sample size population and effect of high dose thiamine on the level of these marker proteins will have to be observed. A large study is resource demanding and hence in which more patients need to be studied for 3–5 years. This would not be justified initially without a small pilot scale trail with surrogate endpoint such as ours is performed. Only then may a large scale resource allocation be justified.

Conflict of interest statement

There is no conflict of interest associated with this research work and project.

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