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Ultraviolet and tandem mass spectrometry for simultaneous quantification of 21 pivotal metabolites in plasma from patients with diabetic nephropathy

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ABSTRACT

A sensitive and specific method was developed for simultaneous determination of 21 compounds related to the diabetic nephropathy (DN) in a single analysis using high-performance liquid chromatography coupled to ultraviolet and tandem mass spectrometry (HPLC–UV/MS/MS) in human plasma. With retention times and MS/MS for peak identification, both UV and MS detectors were used for quantification. Calibration curves suitable for the analysis of plasma were linear ($r^2 > 0.998$) with limits of detection (LOD) from 10 to 1000 ng/mL. Intraday relative standard deviation (R.S.D.) and interday R.S.D. were both lower than 15%. With the case and control study, we found five potential biomarkers of DN, including adenosine, inosine, uric acid, xanthine and creatinine.

1. Introduction

Diabetic nephropathy (DN), the major cause of end-stage renal disease, is a serious complication of diabetes mellitus. It is estimated that the possibility of death due to renal disease is 17 times higher in diabetics than in nondiabetics [1]. In addition, DN is associated with considerably increased risk and mortality of cardiovascular disease. Thus, DN enormously influences the public health. Kim et al. applied proteomics to DN [2,3]. They found out several protein biomarkers of DN, which can be linked to clinical applications [4]. Despite the rapid research progression, molecular markers and diagnostic tools for early and reliable prediction of DN are still insufficient, especially for different phases of DN. Evolution of this project may depend on the understanding of the correlative metabolism.

Purines and pyrimidines are the basic constituents of DNA and RNA. Their synthesis, circulation and catabolism involve a significant number of metabolic processes. Clinical manifestations vary considerably and involve many organ systems, including hematological, immunological and neurological problems, as well as renal disease. It is well established that purine and pyrimidine metabolic pathway is strongly associated with the development of DN. Among related metabolites, adenosine plays an important role in water–electrolyte metabolism, such as renal blood flow, renin release and tubuloglomerular feedback [5,6]. Uric acid, the final product of purine metabolism in humans, may accumulate in patients who are in the status of the end-stage renal disease [7]. There is a controversy that plasma uric acid concentration is a cause or a result of renal disease [8,9]. Considering the complexity of the network and the number of the enzymes involved, a method that quantifies each single metabolite and gives a complete picture of this metabolic compartment would be very useful. Furthermore, creatinine is the major product of phosphocreatine and creatine, which is one of the most widely used markers of renal function. We will quantify them simultaneously in a single analysis. The reference values of the analytes are shown in Table 1.

Numerous methods have been published to detect and quantify creatinine, uric acid, adenosine and other aforementioned metabolites in biological samples, such as high-performance capillary zone electrophoretic assay [18,19], HPLC–UV [12], GC–MS [20,21] and LC–MS/MS [22–25]. However, it was found that all of the methods targeted a small portion of compounds based on literature review, and comprehensive information regarding the metabolic cycles cannot be obtained. In recent studies, la Marca et al. [26] have developed a stable isotope-dilution LC–MS/MS method for the determination of some of them in human urine with excellent analytical performance. However, in order to acquaint ourselves with the full view of purine and pyrimidine related metabolism, there are still several important compounds that need to be investigated.

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Table 1
MRM transitions, declustering potential (DP), collision energy (CE) and quantification strategies chosen for each compound investigated and reference values for each compound.

Measured compound	Precursor ion	Fragment ion	DP (V)	CE (eV)	Quantification strategy	Reference values (μg/mL)
β-alanine	90.2	72.2	90	12	MS	0.19–0.34
Creatine	132.3	90.2	70	18	MS	1–5
Orotic acid	157.3	111.3	50	20	UV	0.02–0.4 [10]
Cytosine	112.1	95	45	25	MS	0.008–1.378
Creatinine	114.2	86.2	40	17	MS	5–15
Dihydrouracil	115.2	55.2	50	27	MS	0.035–0.4 [11]
Uracil	113.2	96	50	25	MS	0.003–0.11 [11]
Uric acid	169.4	141.4	70	20	UV	20–60
Cytidine	244.0	112.2	40	18	UV	No
Hypoxanthine	137.1	110.1	40	18	UV	0.2–0.8 [12]
Uridine	245.2	113.1	50	20	MS	0.115–1 [13]
Xanthine	153.4	110.2	40	15	MS	0.15–0.50 [12]
Thymine	127.1	110.1	40	20	UV	No
Deoxyuridine	229.2	113.1	50	20	UV	0.005–0.14 [14,15]
Inosine	269.2	137.1	50	20	UV	0–0.2 [12]
Guanosine	284.0	152.4	70	25	MS	0.1–0.3 [12]
Deoxyinosine	253.2	137.1	50	20	MS	n.d. [12]
Adenine	136.4	92.2	70	40	UV	0.02–0.2 [16]
Thymidine	243.0	127.2	50	18	UV	0.002–0.02 [17]
Adenosine	268.2	136.2	50	20	UV	0–1.0 [12]
Deoxyadenosine	252.2	136.1	40	20	UV	0–0.15

n.d.: not detectable. No: No reference value.

It is a great challenge to quantify the 21 pivotal metabolites simultaneously because of the following complications: (a) the wide concentration ranges of the analytes–creatinine and uric acid concentrations in body fluids are much higher than the other analytes; (b) some compounds have lower sensitivity with MS detector that cannot be quantified correctly in plasma, such as adenosine, adenine, thymine, thymidine, etc. To the best of our knowledge, no method for the simultaneous quantification of metabolites that cover the 21 analytes has been reported.

Here, we present a selective and sensitive high-performance liquid chromatography tandem mass spectrometry (HPLC–UV/MS/MS) method for simultaneous quantification of 21 relevant metabolites (including creatine, creatinine, orotic acid, β-alanine, cytosine, uracil, dihydrouracil, uric acid, cytidine, hypoxanthine, uridine, xanthine, thymine, deoxyuridine, inosine, guanosine, deoxyinosine, thymidine, adenine, adenosine and deoxyadenosine). As we know, quantification by HPLC–UV needs a good separation because urine and blood usually contain many interfering compounds, while quantification by HPLC–MS/MS may require an internal standard, which is often hard to obtain. Furthermore, some of them have better sensitivity with UV than MS/MS, while the others have better sensitivity with MS/MS than UV. At this point, it is a little bit difficult to quantify as many relevant metabolites as possible simultaneously depend on only one detector. Considering the low LOD and good precision, we decided to use HPLC for plasma sample separation, retention times and MS/MS for peak identification, and both of UV and MS/MS for quantification. Table 1 shows the quantification strategies.

2. Experimental

2.1. Chemicals and reagents

All 21 standards, including creatine, creatinine, orotic acid, β-alanine, cytosine, uracil, dihydrouracil, uric acid, cytidine, hypoxanthine, uridine, xanthine, thymine, deoxyuridine, inosine, guanosine, deoxyinosine, thymidine, adenine, adenosine and deoxyadenosine were purchased from Sigma–Aldrich (St. Louis, MO, USA). HPLC grade methanol was purchased from Fisher Scientific (Fair Lawn, NJ, USA). Analytical grade ammonium acetate and glacial acetic acid was purchased from Beijing Chemical Reagents Company (Beijing, China). Ultra pure water from a Millipore Milli-Q system (Bedford, MA, USA) was used for the preparation of all the solutions.

2.2. Instrumentation

An Applied Biosystems (Toronto, Canada) API 3000 triple quadrupole tandem mass spectrometer was used for HPLC–UV/MS/MS analysis, equipped with a Turbo Ionspray interface and an Agilent 1100 binary HPLC system. The data were processed using Analyst software.

2.3. Standard solution and sample preparation

Plasma samples of 88 DN patients in Beijing, China were collected as cases and 50 plasma samples of healthy people in the same area as controls. They were divided into five groups (control, diabetes without nephropathy (DM), diabetic nephropathy phase III (DN III), diabetic nephropathy phase IV (DN IV) and diabetic nephropathy phase V (DN V)) according to Mogensen. The ages of these people range from 45 to 70 years old. All blood samples were collected into EDTA and centrifuged to obtain plasma in the hospital and sent to our laboratory, where they were stored at −80 °C until sample preparation. All the studied participants had given their informed consent.

Before the start of the analysis, 800 μL of methanol were added to 200 μL aliquots of plasma, vortexed for 2 min, and then centrifuged at 10,000 rpm for 15 min at 4 °C. The clear supernatant was transferred to a 1.5 mL polypropylene tube, and dried under a gentle stream of nitrogen at room temperature. The residue was reconstituted with 100 μL of a mixture of methanol–water (1:1, by volume), and stored at 4 °C before the analysis [12,27].

Working solutions were prepared by diluting the stock solutions of each standard with water, resulting in different concentrations of 50–500 mg/mL. Quality control (QC) samples were prepared by spiking 200 μL aliquots of control plasma with low, medium, and high concentrations of standards to obtain a plasma spiking solution, respectively. All the stock solutions, working solutions and QC

samples were stored at −20 °C and brought to ordinary temperature for use.

2.4. Chromatographic conditions

Samples were separated on an Agilent TC–C_{18} column (250 mm × 4.6 mm I.D., 5 μm particle size, Agilent Technologies) with an Alltech guard column (7.5 mm × 4.6 mm I.D., 5 μm particle size). A mobile phase was used with a flow rate of 0.8 mL/min in which mobile phase A consisted of 10 mM ammonium acetate in Ultra pure water adjusted to pH 5.8 with glacial acetic acid and mobile phase B consisted of 100% methanol. A mobile phase gradient was used starting at 100% A for 5 min, followed by a linear gradient from 100 to 95% A in 5 min, followed by a linear gradient from 95 to 80% A in 10 min, followed by a linear gradient from 80 to 70% A in 20 min, followed by a linear gradient from 40 to 0% A in 5 min. The flow was reduced to 150 μL/min prior to MS detection using a T-split. The column temperature was maintained at 25 °C, the UV detector was set at 254 nm and the injection volume was 20 μL.

2.5. Mass spectrometric conditions

The temperature of the turbo ion electrospray was set at 350 °C. The collision gas (nitrogen) was set at 6 mTorr, and nebulizer gas (nitrogen), curtain gas (nitrogen), and assistant drying gas (air) were used at a flow rate of 8 L/min, 2 L/min, and 4 L/min, respectively. The ion spray voltage was 5000 V. Multiple reaction monitoring (MRM) measurements were performed by using declustering potential (DP) and collision energy (CE) values as manually optimized for each of the analytes. A list of exploited transitions is reported in Table 1. The choice of ionization conditions for each analyte was made with the aim of maximizing the sensitivity under the experimental conditions.

2.6. Calibration and validation

External calibration method was used for the quantitative analysis. Calibration curves were obtained by the plots of the peak area versus the concentration of the standards. The concentrations of the metabolites in plasma samples were determined by using the equations of linear regression obtained from the calibration curves.

Intraday precision (each $n = 5$) were evaluated by the analysis of QC samples spiked with standards of low, medium and high concentration at different times of the same day. Interday precision ($n = 6$) was determined by repeated analysis of QC samples spiked with standards of medium concentration twice per day over three consecutive days. The calibration curves were calibrated everyday by analyzing two working solution samples before analyzing the plasma samples to ensure the precision of the results. Precision was determined by the relative standard deviation (R.S.D.). Signals which are three times higher than the peak noise height were regarded as LOD for the analytes. Because the analytes are endogenous in plasma, LOD and LOQ for them were calculated using the following formula: LOD = 3.3 σ/S; LOQ = 10σ/S. Where σ = the standard deviation of the analytical background response, S = the slope of the calibration curve [28].

The extraction recovery was determined by comparing the peak areas obtained from QC samples with the un-extracted standard working solutions at the same concentration in the same solvent.

The compound stability for 0, 4, 8, 16 and 24 h at −20 °C in plasma was evaluated by repeated analysis at the medium concentration of QC samples.

2.7. Statistical analysis

The mass spectrometry data were processed using Analyst software. Linear regression analysis (Excel) was used to verify the linearity of the calibration curves. Comparisons between cases and controls were done using the Student's t-test. $P < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Optimization of chromatography and mass spectrometry conditions

Extensive chromatographic separation is required for HPLC–UV. Moreover, it can minimize ion suppression from both matrix effects and interferences from co-eluted compounds in the system, so it was essential to increase chromatographic separation. In this study, we evaluated several chromatography columns and combinations of four mobile phases, including ion-pair reagent [15]. The tested HPLC columns included Agilent XDB–C_{18} (5 μm, 4.6 × 250 mm, Agilent Technologies), Waters Xterra–C_{18} (5 μm, 3.9 × 150 mm, Waters), Shiseido–C_{18} (5 μm, 4.9 × 150 mm, Waters), Ultimate AQ–C_{18} (5 μm, 4.6 × 250 mm, Welch Materials Inc.). For the short columns (150 mm), we cannot obtain a good separation of a variety of compounds including adenosine, adenine, thymine, thymidine, etc. And these compounds have lower sensitivity with MS detector that cannot be quantified correctly in plasma without good separation. With Agilent XDB–C_{18} and Ultimate AQ–C_{18} column, the excellent peak shape for alkaline, neutral and acidic compounds was obtained. But UltimateTM AQ–C_{18} had great selectivity and retention capacity only for hydrophilic and polar compounds, such as β-alanine, creatinine and creatine. In our experiment, compared with Agilent XDB–C_{18}, Agilent TC–C_{18} column had a similar or a better separation performance. And it is more applicable to the low proportion of organic phase gradient in the initial stage of analysis. So we decided to complete the separation using Agilent TC–C_{18} column.

And the volatile buffers were ammonium formate, ammonium acetate, formic acid, acetic acid. Retention times, separations, intensities of the analytes, as well as analysis time for each sample were the factors for evaluation. The optimal chromatographic conditions were determined as described in Section 2.4.

To obtain the highest selectivity and lowest limit of quantification, tandem mass spectrometry with MRM mode was used. In order to identify the major species formed in the collisional sequential fragmentation of MS/MS analysis, a mass characterization study was firstly performed for direct infusion (flow rate 10.0 μL/min) of solutions of each compound (10.00 mg/L in methanol). Parameters such as collision energy, capillary voltage, cone voltage, and nitrogen pressure in the collision cell were optimized in both positive and negative ion mode. Most analytes provided better results in positive ion mode. Detailed MS conditions are described in Section 2.5.

Fig. 1 shows the typical UV chromatogram (a) and multiple extracted ion chromatogram (b) of a control plasma fortified with stock solution and subjected to HPLC–UV/MS/MS analysis as described in Section 2.

3.2. Comparison of UV and MS detectors

Both UV and MS detectors can present results for the analysis of these compounds from plasma. In general, the MS detector has higher selectivity and sensitivity, whereas the UV detector is simpler and more accurate with external reference method. In our study, some compounds have higher sensitivity with UV detector,

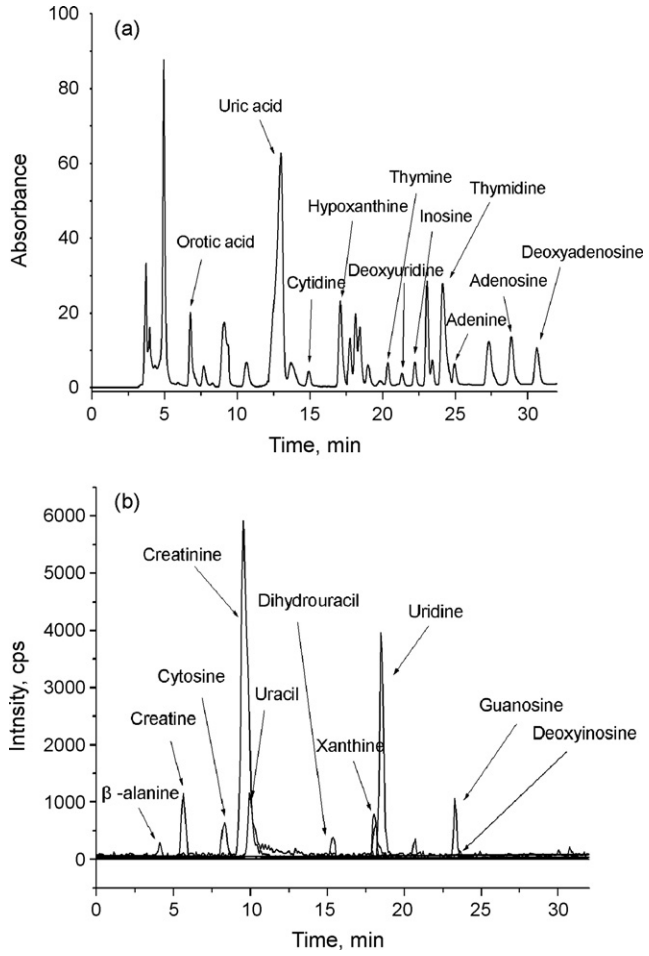

Fig. 1. In a single run, UV chromatogram and multiple extracted ion chromatograms show the separation of pure compounds added to blank plasma. (a) UV chromatogram of 11 compounds; (b) multiple extracted ion chromatograms of other 10 compounds. The added concentrations are: orotic acid, cytidine, thymine: 0.05 μg/mL; deoxyuridine, inosine, thymidine, deoxyadenosine, adenosine, adenine: 0.125 μg/mL; cytosine, hypoxanthine 0.25 μg/mL; creatine, uridine, xanthine, guanosine, deoxyinosine: 1 μg/mL; β-alanine, uracil, dihydrouracil: 5 μg/mL; creatinine: 10 μg/mL; uric acid: 30 μg/mL.

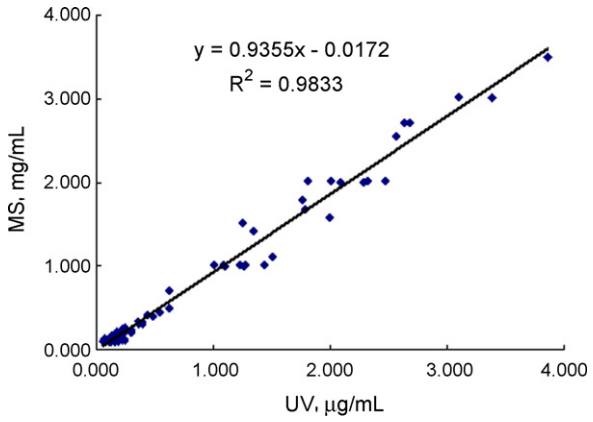

Fig. 2. Comparison of adenosine results obtained by UV and MS detectors.

such as many of purines, pyrimidines and nucleosides. Take adenosine for example, from the structure of adenosine, we can see it has conjugated group that can make it have a strong ultraviolet absorption. As we studied, for adenosine, the LOD was 20 ng/mL with UV detector while 80 ng/mL with MS detector. A slightly lower sensitivity was noticed during the analysis by the MS detector, and it can be improved by using UV detector. The same conclusion was obtained from the analysis of adenine, thymine, thymidine, etc.

The quantification results expressed by UV detector proposed here were compared with MS detector. The quantification results of adenosine by MS detector and UV detector were obtained simultaneously in a single run. Shown in Fig. 2 is a comparison of adenosine results obtained by UV and MS detectors. The comparisons included 90 samples while the other 48 samples had adenosine concentrations below the quantitative limit of the method with MS detector.

3.3. Method validation

The regression equation of calibration curves and their correlation coefficients (r) were calculated as shown in Table 2. All the calibration curves and limits of detection were suitable for the analysis of plasma.

The extraction recoveries were determined at three concentration levels and the results are shown in Table 3. For the endogenous analytes, the background response in blank plasma was subtracted.

Table 2
The Regression equations and limits of detection of 21 compounds.

Measured compound	Regression equation	Linear range (μg/mL)	r^2	LOD (μg/mL)
β-alanine	$y = 17.596x + 763.07$	3–300	0.9985	1
Creatine	$y = 7053.9x + 3410.9$	0.1–10	0.9994	0.04
Orotic acid	$y = 24.901x + 6.1851$	0.02–2	0.9997	0.01
Cytosine	$y = 51360x - 159.07$	0.1–10	0.9992	0.05
Creatinine	$y = 2229.9x + 21.251$	2–200	0.9995	0.5
Dihydrouracil	$y = 12363x + 27521$	1–100	0.9983	0.04
Uracil	$y = 237.43x + 5355.8$	1–100	0.9988	0.6
Uric acid	$y = 33.374x + 13.478$	2–200	0.9998	0.05
Cytidine	$y = 35.214x + 3.3795$	0.02–2	0.9998	0.015
Hypoxanthine	$y = 112.72x + 9.5112$	0.02–2	0.9993	0.02
Uridine	$y = 28.223x + 1.1861$	0.2–20	0.9989	0.08
Xanthine	$y = 225.55x + 45.844$	0.2–20	0.9994	0.1
Thymine	$y = 59.353x + 2.344$	0.02–2	0.9991	0.01
Deoxyuridine	$y = 48.444x + 1.9162$	0.02–2	0.9997	0.015
Inosine	$y = 49.298x + 2.2956$	0.02–2	0.9997	0.015
Guanosine	$y = 2302.5x - 1177.8$	0.2–20	0.998	0.1
Deoxyinosine	$y = 1043.3x - 73.035$	0.2–20	0.998	0.12
Adenine	$y = 33.955x + 1.0626$	0.02–2	0.9998	0.01
Thymidine	$y = 107.37x + 8.2281$	0.02–2	0.9997	0.01
Adenosine	$y = 62.828x + 17.788$	0.02–2	0.9998	0.02
Deoxyadenosine	$y = 66.733x + 4.7737$	0.02–2	0.9996	0.02

Table 3
Precisions and recoveries of spiked QC plasma samples.

Compound	Intraday ($n=5$)						Interday ($n=6$)	Spiked concentration		
	Low[a]		Medium[a]		High[a]		Medium[a]	Low[a] (μg/mL)	Medium[a] (μg/mL)	High[a] (μg/mL)
	Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)	R.S.D. (%)			
β-alanine	97.1	4.99	90.9	7.31	86.7	7.91	8.68	3	5	15
Creatine	84.9	7.75	105.3	6.29	91.8	6.69	8.23	0.3	1	3
Orotic acid	90.5	7.27	102.0	5.04	99.1	3.43	6.17	0.02	0.05	0.15
Cytosine	86.8	9.77	87.4	4.15	93.5	2.63	4.45	0.1	0.25	0.8
Uracil	89.5	8.48	94.7	5.28	98.3	4.64	5.50	3	5	15
Creatinine	86.7	7.75	88.9	5.68	96.8	4.67	7.65	5	50	150
Dihydrouracil	88.4	6.38	92.9	9.44	101.5	4.25	12.4	3	5	15
Uric acid	88.5	3.66	99.2	3.12	99.9	5.25	5.16	20	50	150
Cytidine	99.8	5.84	93.5	4.42	93.2	1.27	5.26	0.02	0.05	0.3
Hypoxanthine	78.6	2.46	89.1	2.38	98.5	4.37	6.68	0.1	0.25	0.8
Uridine	78.9	3.16	102.9	5.52	97.9	1.18	6.62	0.3	1	3
Xanthine	101.2	3.33	101.1	5.57	95.5	3.70	9.01	0.3	1	3
Thymine	94.4	6.63	95.6	3.29	95.7	3.88	6.36	0.02	0.05	0.15
Deoxyuridine	85.2	4.47	90.9	5.01	94.7	3.52	7.50	0.04	0.125	0.4
Inosine	90.1	3.82	100.1	3.41	98.9	1.78	6.08	0.04	0.125	1
guanosine	90.6	5.69	93.4	6.44	94.5	5.68	8.27	0.3	1	3
Deoxyinosine	97.6	5.75	93.7	5.71	99.0	14.9	9.71	0.3	1	3
Thymidine	88.4	5.02	101.6	5.78	102.9	10.4	5.24	0.04	0.125	0.4
Adenine	88.3	8.42	95.6	8.54	96.7	5.70	7.97	0.04	0.125	0.4
Adenosine	93.3	7.29	96.7	3.57	95.3	3.49	4.68	0.04	0.125	0.4
Deoxyadenosine	86.6	2.16	99.5	6.69	97.0	3.84	5.72	0.04	0.125	0.4

[a] Plasma samples spiked with low, medium, or high concentrations.

The data from QC samples were calculated to estimate the intraday precision, interday precision and compounds stability of the method. Intraday relative standard deviation (R.S.D.) and interday R.S.D. were both lower than 15%. Compounds stability relative standard deviation (R.S.D.) was lower than 10%. The result showed good stability of these 21 components. The detailed results are listed in Table 3.

3.4. Case–control study

The present method was applied to analyze the plasma samples of cases and controls. Fifteen compounds were observed in plasma samples to the detection limit of this assay. All of the 6 other compounds were not observed. According to the reference values, for several compounds, the LOQ for the method is high. On the one hand, the range of reference value is wide for some compounds, so we cannot detected the compounds in many samples, such as dihydrouracil, guanosine and deoxyadenosine. On the other hand, our method is for the overall screening of metabolites revolved in purine and pyrimidine metabolism, so it is difficult for us to obtain low LOD for some compounds, such as β-alanine, uracil and deoxyinosine.

Fig. 3 shows the difference between a typical healthy subject and a typical DN V patient. The concentrations of the analytes in these two samples are shown in Table 4. The total results of all subjects are illustrated in Table 5. In Table 5, we can see that the levels of uric acid, xanthine and inosine in the group of DN V were significantly higher as compared with DN IV and control group. For level of adenosine, statistically significant differences between the groups of DM and DN III, DN IV and DN V were observed. And the level of creatinine increases gradually with the aggravation of DN.

In the kidney, adenosine plays an important role in water–electrolyte metabolism, such as in the glomerular filtration rate, renal blood flow, renin release, tubuloglomerular feedback, tubular sodium and water transport, and neurotransmitter release. The extracellular metabolism of adenosine is mediated by two mechanisms. First, adenosine is taken up quickly and efficiently by red blood cells, via an equilibrative facilitated diffusion system [29]. Second, adenosine is deaminated rapidly into inosine by adenosine deaminase (ADA). ADA is found in large amounts particularly in mononuclear cells [30,31], where it plays a major role in adenosine concentration regulation in both extracellular and intracellular spaces [32,33]. Some researchers had suggested that it was mediated by a decrease in the activity and expression of ADA, increased production of adenosine, and an induced imbalance in adenosine receptors. Moreover, type II DM is characterized by insulin resistance, a failure of the beta cell to produce enough insulin to overcome the resistance. The deficiency of insulin may lead to inappropriate immunoresponses even immune defect. A normal ADA activity level prevents adenosine (a strong immunosuppressive agent [34,35]) accumulation and thus ensures normal lymphocyte development and function [36]. The decrease in ADA activity, which induces high adenosine concentrations in body fluids, causes severe combined immunodeficiency syndrome. The results have indicated that dramatic increasing of adenosine probably accelerates the progression of DN and toxuria.

Additionally, uric acid has various physiologic functions, including an inhibition of endothelial nitric oxide bioavailability, slow renal blood flow, activation of the rennin angiotensin system, and direct actions on endothelial cells and vascular smooth muscle cells. Hyperuricemia also has been found to accelerate renal disease and then damage the nephric tubule irreversibly. Moreover,

Table 4
Quantification results of a typical healthy subject and a typical DN V patient.

Compound	Control	DN V
Creatine (μg/mL)	0.832	1.243
Orotic acid (μg/mL)	0.024	0.069
Cytosine (μg/mL)	0.108	0.454
Creatinine[a,b,c,d] (μg/mL)	8.451	87.432
Uric acid[a,d] (μg/mL)	42.323	79.862
Cytidine (μg/mL)	0.063	0.187
Hypoxanthine (μg/mL))	0.306	0.377
Uridine (μg/mL)	1.317	0.906
Xanthine[d] (μg/mL)	0.503	1.754
Thymine (μg/mL)	0.025	0.187
Deoxyuridine (μg/mL))	0.072	0.362
Inosine[d] (μg/mL)	0.079	0.647
Adenine (μg/mL)	0.102	0.387
Thymidine (μg/mL)	0.031	0.129
Adenosine[b,d] (μg/mL)	0.126	1.253

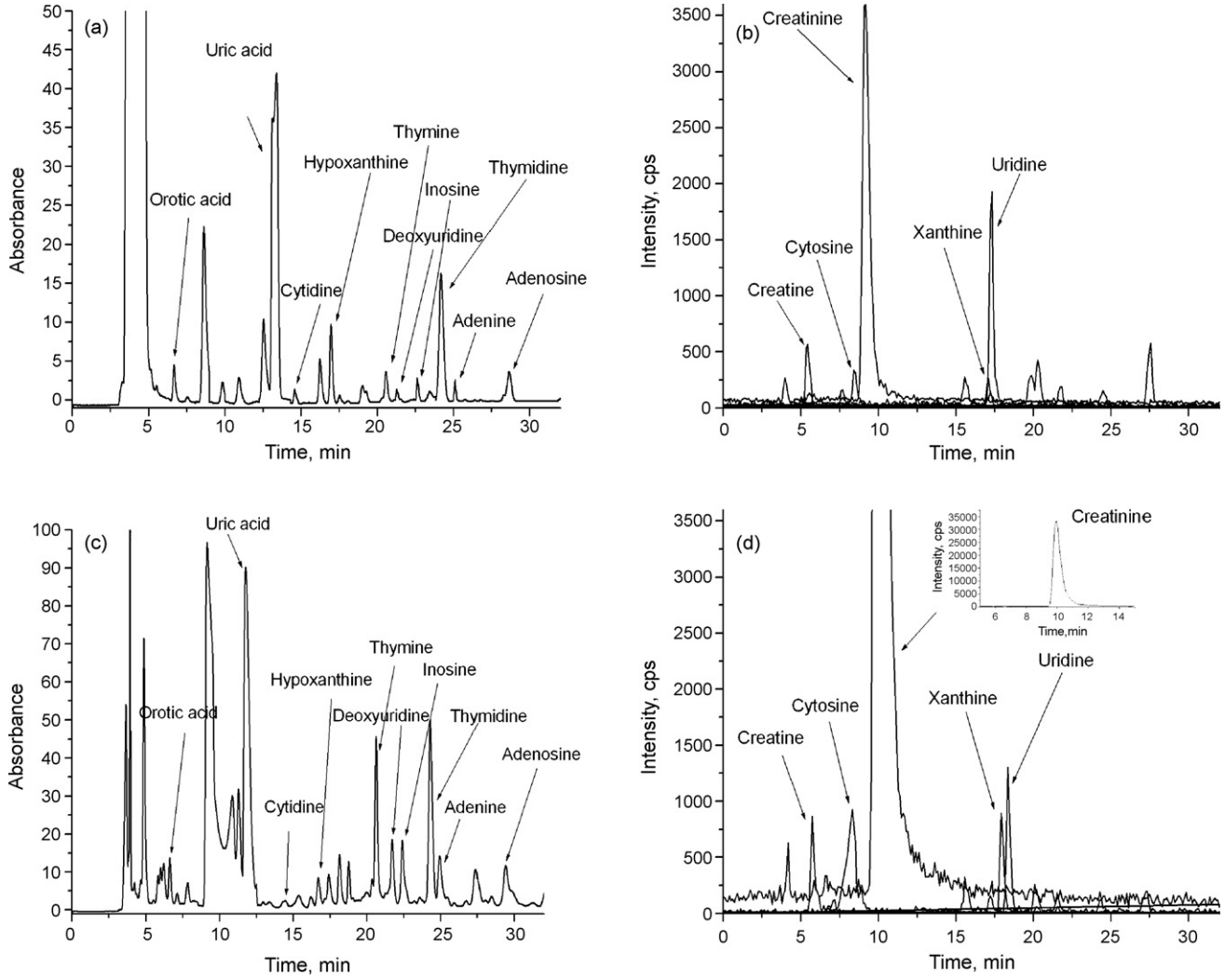

Fig. 3. UV chromatogram and multiple extracted ion chromatograms of 15 compounds in plasma of a control subject and a DN V patient. (a) UV chromatogram of 10 compounds in plasma of a control subject; (b) multiple extracted ion chromatograms of 5 compounds in plasma of a control subject; (c) UV chromatogram of 10 compounds in plasma of a DN V patient; (d) multiple extracted ion chromatograms of 5 compounds in plasma of a DN V patient.

Table 5
Quantification results of plasma samples.

Compound	Control (mean ± *s*, *n* = 50)	DM (mean ± *s*, *n* = 27)	DN III (mean ± *s*, *n* = 17)	DN IV (mean ± *s*, *n* = 16)	DN V (mean ± *s*, *n* = 28)
Creatine (μg/mL)	1.226 ± 0.282	0.911 ± 0.207	0.504 ± 0.124	0.575 ± 0.149	0.975 ± 0.213
Orotic acid (μg/mL)	0.021 ± 0.003	0.030 ± 0.005	0.033 ± 0.005	0.023 ± 0.010	0.085 ± 0.025
Cytosine (μg/mL)	0.115 ± 0.015	0.194 ± 0.038	0.223 ± 0.077	0.305 ± 0.072	0.520 ± 0.167
Creatinine[a,b,c,d] (μg/mL)	7.321 ± 0.770	11.282 ± 1.447	19.080 ± 5.209	53.360 ± 14.229	82.101 ± 11.889
Uric acid[a,d] (μg/mL)	46.527 ± 3.060	58.624 ± 5.656	61.555 ± 8.632	63.867 ± 8.871	76.450 ± 5.651
Cytidine (μg/mL)	0.046 ± 0.012	0.051 ± 0.007	0.055 ± 0.011	0.074 ± 0.024	0.296 ± 0.069
Hypoxanthine (μg/mL))	0.287 ± 0.036	0.283 ± 0.075	0.200 ± 0.090	0.218 ± 0.070	0.528 ± 0.231
Uridine (μg/mL)	1.226 ± 0.096	1.394 ± 0.168	0.934 ± 0.165	1.235 ± 0.212	1.119 ± 0.177
Xanthine[d] (μg/mL)	0.477 ± 0.062	0.554 ± 0.159	0.463 ± 0.129	0.439 ± 0.199	2.034 ± 0.657
Thymine (μg/mL)	0.035 ± 0.008	0.030 ± 0.007	0.035 ± 0.015	0.029 ± 0.007	0.077 ± 0.023
Deoxyuridine (μg/mL))	0.194 ± 0.055	0.099 ± 0.027	0.065 ± 0.016	0.122 ± 0.052	0.162 ± 0.056
Inosine[d] (μg/mL)	0.077 ± 0.012	0.080 ± 0.015	0.084 ± 0.023	0.119 ± 0.100	0.947 ± 0.346
Adenine (μg/mL)	0.165 ± 0.036	0.146 ± 0.055	0.159 ± 0.064	0.170 ± 0.119	0.187 ± 0.042
Thymidine (μg/mL)	0.028 ± 0.005	0.039 ± 0.012	0.071 ± 0.034	0.114 ± 0.054	0.339 ± 0.093
Adenosine[b,d] (μg/mL)	0.136 ± 0.030	0.138 ± 0.029	0.195 ± 0.073	0.240 ± 0.080	1.870 ± 0.407

[a] $P < 0.05$ between control and DM.
[b] $P < 0.05$ between DM and DN III.
[c] $P < 0.05$ between DN III and DN IV.
[d] $P < 0.05$ between DN IV and DN V.

increased uric acid level will induce the oxidation of low density lipoprotein cholesterol and peroxidation of lipid. Hence, strategies to control and decrease plasma uric acid levels may have a beneficial effect on improving kidney function or slowing the progression of renal diseases in clinical practice. Xanthine acts as a substrate for xanthine oxidase and enhances superoxide generation. When xanthine oxidase converts hypoxanthine to xanthine in the presence of molecular oxygen, superoxide radicals ($O_2^{\bullet -}$) are released. Reactive oxygen species (ROS) generated during the progression of DN play a major role in microvascular dysfunction and exert direct tissue damage, leading to lipid peroxidation, denaturation of proteins, and oxidation of DNA [30]. Many direct evidences have demonstrated that ROS was one of the most important mechanisms of DN.

As seen in Table 5, at the phase of DN V, there is a significant increase of adenosine, inosine, uric acid, xanthine and creatinine. The reason is that DN V is a phase of renal failure, thus severe metabolic disorder has appeared. The results suggested that these compounds may be the potential biomarkers for the diagnosis of progression of DN.

4. Conclusion

In this study of 21 purine and pyrimidine compounds in the plasma of diabetes patients and DN patients, we used HPLC for plasma sample separation, retention times and MS/MS for peak identification, then UV and MS/MS detection for quantification. Ultraviolet detection has higher stability and lower LOD for some of the analytical compounds. Triple quadrupole tandem mass spectrometry with MRM brings high selectivity and high sensitivity. The use of the both techniques makes it possible to quantify as many metabolites as possible in complex biological samples simultaneously. Although MRM may lead to a loss of information of substances other than the targets, it can provide enough information for peak identification and accurate quantitative information of pivotal substances in the system.

The presented method provided a reliable way in quantifying most of the compounds involved in the purine and pyrimidine metabolism simultaneously, thus established the foundation of research for mechanism elucidation of DN. As a result, it is now possible to identify those with multiple risk factors of DN rather than one single risk factor. In conclusion, the presented method and findings will provide a solid foundation for prenatal diagnosis and prevention of DN, as well as some other purine and pyrimidine metabolism related diseases such as gout and coronary heart disease.

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