

METHOD FOR SIMULTANEOUSLY DETECTING OXALIC ACID, CITRIC ACID AND CYSTINE IN 24H URINE AND USE THEREOF

TECHNICAL FIELD

[0001] The present disclosure relates to the technical field of detection, particularly to a method for simultaneously detecting oxalic acid, citric acid and cystine in 24h urine and use thereof.

BACKGROUND

[0002] Nephrolithiasis is a common and high incidence disease. At present, the morbidity of nephrolithiasis in China is 5%-10%, and the recurrence rate of nephrolithiasis in 5 years is as high as 70%-80%. The incidence of nephrolithiasis can cause urinary tract infection, waist and abdominal pain, hydronephrosis, renal failure and the like, and meanwhile repeated medical and surgical treatment brings huge economic and living burden for patients. At present, there have been mature schemes for diagnosis and treatment of nephrolithiasis. However, how to effectively reduce the recurrence of nephrolithiasis is still a major problem in clinic.

[0003] The 24h urine metabolism assessment is recommended by the current domestic and foreign urological guidelines, can be applied to patients with complex calculus (patients with recurrent calculus and residual calculus in kidney, and patients with risk factors), and provides a systematic and comprehensive metabolic assessment for the patients, so as to formulate an effective plan to prevent the recurrence of calculus. 24h urine assessment refers to collecting all urines of nephrolithiasis patients in 24 h and detecting the levels of key metabolites of patients in 24h urine, such as oxalic acid, citric acid, cystine, uric acid, creatinine, calcium, phosphorus and potassium.

[0004] In 24 h urine detection indexes, oxalic acid, citric acid and cystine are important metabolic indexes. Calcium oxalate (CaOx) is the most common calculus component accounting for about 80% or more of nephrolithiasis. Urinary oxalic acid together with calcium ions in urine easily forms CaOx, and therefore the level of urinary oxalic acid plays an important role in the formation of kidney calculus. On the other hand, citric

acid is an important protective factor to prevent the formation of kidney calculus, which can prevent the formation of kidney calculus from multiple mechanisms. Citric acid together with calcium ions can form a soluble complex, thus inhibiting the formation of crystals in urine and supersaturation of calcium salts. Hypocitraturia is a common feature of patients with primary kidney calculus, especially those with calcium-containing calculus. Cystine calculus is a special type of kidney calculus, which is formed by a fact that congenital genetic variation leads to abnormal cystine metabolism so as to cause cystinuria to induce deposition of insoluble cystine in urine. It can be seen that the detection of these 3 metabolites plays an important role in the metabolic assessment of nephrolithiasis. However, by integrating the domestic and abroad literatures and laboratory detection reports, there are still no products simultaneously and accurately detecting oxalic acid, citric acid and cystine by using a method.

[0005] At present, it is reported at home and abroad that in metabolite detection of 24h urine, oxalic acid and citric acid are detected by ion chromatograph or ultraviolet spectrophotometer enzyme method in most cases, while substances calcium, phosphorus, potassium and sodium are mainly detected by using an automatic biochemical analyzer. Compared with the above methods, mass spectrometric detection is considered to be a higher-order more accurate method. Th detection of oxalic acid, citric acid and cystine in 24h urine by mass spectrometry is more accurate than the enzyme method and ion chromatography. Meanwhile, considering that oxalic acid and citric acid have the chemical characteristic of poor stability, the mass spectrometric detection method is simpler in operation process than the enzyme method and the ion chromatography, and also reduces errors caused in the processing process.

[0006] At present, the methods for detecting metabolites in urine at home and abroad have the following problems:

[0007] 1. At present, most of the technical patents fail to realize the simultaneous and accurate detection of the key metabolites of kidney calculus-oxalic acid, citric acid and cystine, and difficultly achieves a rapid and comprehensive 24h urine metabolism assessment.

[0008] 2. The process required for currently reported patented technology for the detection of oxalic acid and citric acid by urine mass spectrometry is still complicated, takes a long time and is high in cost, and therefore large-scale automation and marketization are difficult to achieve.

[0009] 3. The current detection technology patent has not clearly explored the stability of metabolites in 24h urine, especially the effect on metabolites such as oxalic acid and citric acid in 24h urine after different preservatives are added.

[0010] Therefore, clinically, it is urgent to develop a method that can simultaneously detect oxalic acid, citric acid and cystine in 24h urine, which is simple, rapid and good in repeatability, and products produced by using the method.

SUMMARY

[0011] The present disclosure provides a method for simultaneously detecting oxalic acid, citric acid and cystine in 24h urine and use thereof, aiming at solving the above problems existing in the above background technology.

[0012] In order to realize the above technical objective, the present disclosure mainly adopts the following technical solution:

[0013] Provided is a method for simultaneously detecting oxalic acid, citric acid and cystine in 24h urine, comprising the following steps:

[0014] S1, preparation of a test sample: taking a to-be-detected 24h urine sample, and adding oxalic acid-1,2-¹³C₂ as an internal standard of oxalic acid, citric acid-2,2,4,4-d₄ as an internal standard of citric acid and L-cystine-d₄ as an internal standard of cystine; and performing derivation treatment followed by successively adding a tetramethyl ammonium fluoride solution and an acetonitrile solution to be evenly mixed, then injecting the mixed solution onto a weak cation exchange 96-well plate, and collecting eluent as the test sample; and

[0015] S2, detection: detecting the test sample utilizing a liquid chromatography tandem mass spectrometer.

[0016] Wherein, in step S1, the derivation treatment comprises the following steps: successively adding benzyl alcohol and trimethylchlorosilane into a 24h to-be-detected

urine sample added with an internal standard working solution to be evenly mixed by vortex vibration, and then putting the obtained mixture in an oven to be derived at 80°C.

[0017] Preferably, in the internal standard solution, the concentration of oxalic acid-1,2-¹³C₂ is 25 µg/mL, the concentration of citric acid-2,2,4,4-d₄ is 10 µg/mL, and the concentration of L-cystine-d₄ is 12.5 µg/mL.

[0018] In step S2, the detection conditions of liquid chromatography are as follows:

[0019] mobile phase A of a high pressure pump of a liquid chromatography system:

0.1% formic acid-2 mM ammonium acetate-aqueous solution;

[0020] mobile phase B of a high pressure pump of a liquid chromatography system:

0.1% formic acid-2 mM ammonium acetate-methanol solution;

[0021] a gradient elution program is as follows:

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0.00	60	40
0.50	60	40
1.00	25	75
2.60	25	75
2.70	0	100
3.40	0	100
3.50	60	40
4.00	60	40

[0022] Further, in chromatographic conditions, a chromatographic column is henomenex F5 Column, 2.6 µm, 100A, 3.0×50 mm, and the flow rate of the mobile phase is 0.6 mL/min.

[0023] Still further, in chromatographic conditions, the column temperature is 40°C, and the injection amount is 5 µL.

[0024] In addition, in step S2, mass spectrum conditions are as follows: an electrospray ionization source, a positive ion mode, spraying voltage: 5500V; temperature: 500°C; atomization air: 55 psi; auxiliary heating air: 55 psi; air curtain: 25 psi; Collision activated dissociation (CAD): 9; specific monitoring ion pairs and collision energy are as follows:

Analyte	Q1Mass	Q3Mass	Time (msec)	DP	CE	CXP	Corresponding internal standard
Dibenzyl Oxa-1	288.1	108.1	30	82	13	12	Dibenzyl

							Oxa-13C2-1
Dibenzyl Oxa-2	288.1	181.0	15	82	11	11	Dibenzyl Oxa-13C2-2
Tribenzyl Cit-1	463.1	237.1	30	75	12	7	Tribenzyl Cit-d4-1
Tribenzyl Cit-2	463.1	283.1	15	75	10	9	Tribenzyl Cit-d4-2
Dibenzyl Cys-1	421.1	210.1	30	110	27	12	Dibenzyl Cys-d4-1
Dibenzyl Cys-2	421.1	300.1	15	110	24	9	Dibenzyl Cys-d4-2
Dibenzyl Oxa-13C2-1	290.1	108.1	30	80	13	12	
Dibenzyl Oxa-13C2-2	290.1	181.0	15	80	12	11	
Tribenzyl Cit-d4-1	467.2	241.1	30	75	12	7	
Tribenzyl Cit-d4-2	467.2	287.1	15	75	10	9	
Dibenzyl Cys-d4-1	425.2	212.1	30	110	27	12	
Dibenzyl Cys-d4-2	425.2	302.1	15	110	24	9	

[0025] As one preferred embodiments of the present disclosure, the detection method of the present disclosure also comprises: using stable isotope labeled oxalic acid-1,2-¹³C₂ as an internal standard of oxalic acid, citric acid-2,2,4,4-d₄ as an internal standard of citric acid and L-cystine-d₄ as an internal standard of cystine; plotting a standard curve by preparing a series of standard products with different concentrations, performing linear regression analysis by using the concentration of a standard solution as X axis and using a ratio of standard and internal standard peak areas as Y axis, and obtaining a regression equation via “1/X” weight; and substituting a ratio of a peak area of a to-be-detected component in the sample to an peak area of an internal standard of the component into the standard curve equation to calculate concentrations of to-be-detected components in the urine sample.

[0026] As one preferred embodiments of the present disclosure, the detection method of the present disclosure also comprises: measuring background values of oxalic acid, citric acid and cystine in an artificial matrix, adding known concentrations of oxalic acid, citric acid and cystine to the artificial matrix, detecting by a liquid chromatography tandem mass spectrometer, and comparing actually measured oxalic acid, citric acid and cystine with theoretically calculated oxalic acid, citric acid and cystine to judge the reliability of the liquid chromatography tandem mass spectrometer.

[0027] Another objective of the present disclosure is to provide use of a kit in urine

metabolism assessment of a nephrolithiasis patient, wherein the kit comprises a marker composition consisting of oxalic acid, citric acid and cystine, and the marker composition is detected by using the method for simultaneously detecting oxalic acid, citric acid and cystine in 24h urine as described above.

[0028] Compared with the prior art, the present disclosure has the following beneficial effects:

[0029] (1) Compared with the direct detection method, a method for first deriving organic acid in urine and then detecting takes a long time, but is more steady and more accurate in detection. Meanwhile, through the derivation reaction, the polarity of the compound is significantly lowered, which is more conducive to separating a citric acid-isocitric acid isomeride so that detection results are more accurate.

[0030] (2) The derivation method used in the present disclosure is conducted in the water environment. For biological samples rich in water, such as urine, the pretreatment process is more convenient, and the reaction can be carried out without blow drying. Meanwhile, the requirements for the humidity of the external environment are reduced. The high-humidity environment does not affect the derivation effect, and the method is more stable.

[0031] (3) The whole process does not need nitrogen blowing and plate replacement, which can not only save economic cost, but also help to realize automation in the later stage.

DETAILED DESCRIPTION OF THE EMBODIMENTS

[0032] The following examples are used for illustrating the present disclosure, but not limiting the scope of the present disclosure. Where no specific technology or condition is specified in the embodiments, the technology or condition described in the literature in the art or the product specification shall be followed. If the manufacturer is not specified for the reagents or instruments used, they are conventional products that can be purchased through regular channels.

[0033] Example 1

[0034] Detection of oxalic acid, citric acid and cystine in 24h urine using the

method of the present disclosure.

[0035] 1.1 Preparation of reagent

[0036] 1.1.1 Preparation of standard solution

[0037] 1.1.1.1 Preparation of standard stock solution, see in Table 1

Table 1 Preparation of standard stock solution

Analyte	Weight (mg)	Conversion factor	Water (μL)	1N HCl solution (μL)	Concentration of stock solution (mg/mL)
Oxalic acid	10.27	1.002	1029		10
Citric acid	24.33	0.998	809		30
Cystine	11.10	0.999		1108	10

[0038] 1.1.1.2 Preparation of mixed working stock solution (Mix), see in Table 2

Table 2 Preparation of mixed working stock solution (Mix)

Stock solution	Standard product	Concentration of stock solution (mg/mL)	Addition amount of stock solution (μL)	Addition amount of PBS solution (μL)	Concentration (mg/mL)
Mix	Oxalic acid	10	100	700	1
	Citric acid	30	100		3
	Cystine	10	100		1

Note: 1) the volume can be properly adjusted on the premise of keeping the ratio unchanged.

2) The standard stock solution and the mixed stock solution were placed in a refrigerator at -80°C.

[0039] 1.1.1.3 Preparation of standard curve samples, and specific concentrations are seen in Table 3. Standard curve samples were sub-packaged and then cryopreserved.

Table 3 Standard curve concentration of each analyte

Number	Analyte	Standard curve concentration (μg/mL)
1	Oxalic acid	1, 5, 10, 50, 100, 200
2	Citric acid	3, 15, 30, 150, 300, 600
3	Cystine	1, 5, 10, 50, 100, 200

[0040] 1.1.2 Preparation of Self-quality control (QC) Products

[0041] 1.1.2.1 Detection of background values: an artificial urine matrix was taken, the

background values of the contents of oxalic acid, citric acid and cystine in the matrix were measured according to the conventional detection process, the background sample was divided into three parts, and then different amounts of oxalic acid, citric acid and cystine with known concentrations were respectively added into the three artificial matrix samples as low-concentration quality control (LQC), medium-concentration quality control (MQC), and high-concentration quality control (HQC) products. The actually measured oxalic acid, citric acid and cystine were compared with the theoretically calculated oxalic acid, citric acid and cystine in quality control low-concentration quality control (LQC), medium-concentration quality control (MQC) and high-concentration quality control (HQC) through a liquid chromatography tandem mass spectrometer, so as to judge the reliability of the detection method with the liquid chromatography tandem mass spectrometer.

[0042] Wherein, preparation methods of quality control low-concentration quality control (LQC), medium-concentration quality control (MQC) and high-concentration quality control (HQC) are as shown in Table 4.

Table 4 Preparation of Quality control (QC) products

Quality control (QC) products	Addition amount of mixed stock solution (μL)	Addition amount of artificial urine (μL)	Total volume (μL)
LQC	50	950	1000
MQC	20	980	1000
HQC	100	900	1000

[0043] The concentrations of Quality control (QC) products prepared according to Table 4 are as shown in Table 5.

Table 5 Preparation concentrations of Quality control (QC) products

Quality control (QC) products	Concentration of Quality control (QC) products ($\mu\text{g/mL}$)		
	Oxalic acid	Citric acid	Cystine
LQC	$5+0.95 C_{\text{background}}$	$15+0.95 C_{\text{background}}$	$5+0.95 C_{\text{background}}$
MQC	$20+0.98 C_{\text{background}}$	$60+0.98 C_{\text{background}}$	$20+0.98 C_{\text{background}}$
HQC	$100+0.9 C_{\text{background}}$	$300+0.9 C_{\text{background}}$	$100+0.9 C_{\text{background}}$

Note: the volume can be properly adjusted on the premise of keeping the ratio unchanged.

[0044] 1.1.3 Preparation of internal standard (IS) Solution

[0045] 1.1.3.1 Preparation of internal standard stock solution, see Table 6.

Table 6 Preparation of internal standard stock solution

Internal standard	Weight (mg)	Conversion factor	water (μL)	1N HCl (μL)	Acetonitrile (μL)	Concentration(mg/mL)
Oxalic acid-1,2- ¹³ C ₂	10.95	1			1095	10
Citric-2,2,4,4-d ₄	10	1	1000			10
L-cystine-d ₄	1	1		1000		1

[0046] 1.1.3.2 Preparation of internal standard working solution, see Table 7

Table 7 Preparation of internal standard (IS) working solution

internal standard (IS)	Addition volume of internal standard (IS) stock solution (μL)	Addition volume of 60% acetonitrile (μL)	Concentration of internal standard working solution (μg/mL)
Oxalic acid-1,2- ¹³ C ₂	25	9840	25
Citric acid-2,2,4,4-d ₄	10		10
L-cystine-d ₄	125		12.5

Note: the internal standard (IS) stock solution was stored in a refrigerator at -80°C, and the internal standard (IS) working solution was stored in a refrigerator at -20°C.

[0047] 1.1.4 Preparation of other solutions

[0048] 1.1.4.1 1N hydrochloric acid (HCl) solution: 1 mL of concentrated hydrochloric acid (HCl) was 9 mL of water mixed.

[0049] 1.1.4.2 PBS solution: 1 mL of PBS (10 ×) solution was taken into 9 mL of water to be evenly mixed.

[0050] 1.1.4.3 Tetramethyl ammonium fluoride solution (0.3mM): 2.79 mg of tetramethyl ammonium fluoride solid was weighed to be dissolved into 1 mL of isopropanol to obtain 30 mM tetramethyl ammonium fluoride mother liquor which was stored in a refrigerator at -20°C. When in use, 100 μL of mother liquor was dissolved into 10 mL of isopropanol (diluted by 100 times) to obtain 0.3mM tetramethyl ammonium fluoride solution.

[0051] 1.2 Pretreatment

[0052] a) 20 μL of double blank (PBS solution), single blank (PBS solution), a standard curve, a quality control (QC) sample and a urine sample were successively sucked to be

added to 1.5 mL of labeled EP tube respectively; wherein, double blank refers to no standard and no internal standard, and single blank refers to no standard and no internal standard;

[0053] b) 20 μ L of 60% Acetonitrile (ACN) was added into the double blank sample, and 20 μ L of internal standard working solution was added into other samples;

[0054] c) 50 μ L of benzyl alcohol was added into all samples and then 30 μ L of trimethylchlorosilane was added, the above materials were vibrated by vortex for 2 min;

[0055] d) the above obtained mixture was put into an oven and derived at 80°C for 75 min;

[0056] e) After taking, 40 μ L of tetramethylammonium fluoride solution was added and then 200 μ L of 80% acetonitrile solution was added, and the above materials were vibrated by vortex for 2 min; and

[0057] f) 100 μ L of treated solution was taken to be added into a 96-well injection plate.

[0058] 1.3 Detection

[0059] Liquid chromatography (LC) Conditions

[0060] Mobile phase A of a high pressure pump of a liquid chromatography system: 0.1% formic acid-2 mM ammonium acetate aqueous solution

[0061] Mobile phase B of a high pressure pump of a liquid chromatography system: 0.1% formic acid-2 mM ammonium acetate methanol solution

[0062] Flow rate: 0.6 ml / min

[0063] Chromatographic column: phenomenex F5 column, 2.6 μ m, 100A, 3.0 $\times$ 50 mm

[0064] Column temperature: 40°C

[0065] Injection volume: 5 μ L

[0066] Gradient elution parameters are as follows:

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0.00	60	40
0.50	60	40
1.00	25	75
2.60	25	75
2.70	0	100

3.40	0	100
3.50	60	40
4.00	60	40

[0067] It can be seen from a detection spectrogram that retention time: citric acid: 2.54 min; oxalic acid: 1.99 min; cystine: 1.36 min

[0068] 1.4 During the redetection, the mass spectrometry (MS) conditions used in combination are as follows:

[0069] Electrospray ionization (ESI) source, positive ion mode;

[0070] Spray voltage: 5500 V;

[0071] Temperature: 500°C;

[0072] Atomizing gas: 55 psi;

[0073] Auxiliary heating gas: 55 psi;

[0074] Air curtain: 25 psi;

[0075] CAD: 9

[0076] Multiple reaction monitoring (MRM) scanning mode;

[0077] ion pair

Analyte	Q1Mass	Q3Mass	Time (msec)	DP	CE	CXP	Corresponding internal standard
Dibenzyl Oxa-1	288.1	108.1	30	82	13	12	Dibenzyl Oxa-13C2-1
Dibenzyl Oxa-2	288.1	181.0	15	82	11	11	Dibenzyl Oxa-13C2-2
Tribenzyl Cit-1	463.1	237.1	30	75	12	7	Tribenzyl Cit-d4-1
Tribenzyl Cit-2	463.1	283.1	15	75	10	9	Tribenzyl Cit-d4-2
Dibenzyl Cys-1	421.1	210.1	30	110	27	12	Dibenzyl Cys-d4-1
Dibenzyl Cys-2	421.1	300.1	15	110	24	9	Dibenzyl Cys-d4-2
Dibenzyl Oxa-13C2-1	290.1	108.1	30	80	13	12	
Dibenzyl Oxa-13C2-2	290.1	181.0	15	80	12	11	
Tribenzyl Cit-d4-1	467.2	241.1	30	75	12	7	
Tribenzyl Cit-d4-2	467.2	287.1	15	75	10	9	
Dibenzyl Cys-d4-1	425.2	212.1	30	110	27	12	
Dibenzyl Cys-d4-2	425.2	302.1	15	110	24	9	

[0078] Example 2

[0079] The detection method in example 1 was verified. The results are as follows.

[0080] 2.1 Accuracy and within-run precision

Citric acid	Theoretical value	Measurement value	Accuracy (%)	Average	SD	CV %	Bias %
Level 1	11.27	12.8200	113.74	12.02	0.55	4.59	6.65
		11.5700	102.64				
		12.3600	109.68				
		11.5700	102.67				
		11.7800	104.50				
Level 2	87.23	94.1800	107.97	87.63	4.21	4.80	0.45
		88.3400	101.28				
		87.4700	100.28				
		85.1000	97.56				
		83.0400	95.20				
Level 3	210.72	222.1700	105.43	223.94	9.22	4.12	6.28
		238.8500	113.35				
		216.3300	102.66				
		216.6100	102.80				
		225.7600	107.14				

Oxalic acid	Theoretical value	Measurement value	Accuracy (%)	Average	SD	CV %	Bias %
Level 1	2.63	2.6800	102.00	2.72	0.07	2.75	3.35
		2.8100	106.84				
		2.6900	102.29				
		2.6300	100.08				
		2.7800	105.65				

Level 2	32.87	33.8200	102.90	33.82	0.89	2.65	2.90
		34.0800	103.68				
		35.1700	107.01				
		32.8700	100.00				
		33.1800	100.95				
Level 3	111.82	111.8700	100.05	113.40	2.81	2.48	1.41
		113.6200	101.61				
		117.1900	104.80				
		109.7300	98.13				
		114.5700	102.46				

Cystine	Theoretical value	Measurement value	Accuracy (%)	Average	SD	CV %	Bias %
Level 1	3.11	3.3400	107.46	3.10	0.14	4.61	-0.32
		2.9900	96.01				
		3.1200	100.25				
		3.0400	97.76				
		3.0100	96.68				
Level 2	35.24	37.3200	105.89	36.62	1.22	3.34	3.91
		37.4600	106.31				
		37.3800	106.07				
		34.5900	98.15				
		36.3400	103.11				
Level 3	114.00	121.7800	106.82	121.04	3.59	2.97	6.17

		122.5400	107.49				
		115.8000	101.58				
		119.6300	104.94				
		125.4400	110.03				

[0081] 2.2 Within-run precision

Citric acid	Theoretical value	Measurement value			Average	SD	CV %
Level 1	11.27	12.82	10.95	11.08	11.33	0.64	5.69
		11.57	11.07	10.59			
		12.36	11.12	10.50			
		11.57	11.71	10.84			
		11.78	11.17	10.83			
Level 2	87.23	94.18	91.27	86.37	86.87	3.56	4.10
		88.34	89.85	82.35			
		87.47	83.27	82.62			
		85.10	90.35	83.67			
		83.04	87.28	87.86			
Level 3	210.72	222.17	215.17	199.01	215.05	11.73	5.46
		238.85	222.43	228.05			
		216.33	208.13	208.69			
		216.61	210.44	191.22			
		225.76	209.55	213.41			

Oxalic acid	Theoretical value	Measurement value			Average	SD	CV %
Level 1	2.63	2.68	2.80	2.60	2.65	0.13	5.02
		2.81	2.70	2.43			
		2.69	2.63	2.51			
		2.63	2.73	2.40			
		2.78	2.80	2.53			
Level 2	32.87	33.82	37.69	32.08	34.38	2.76	8.01
		34.08	38.63	29.47			
		35.17	36.69	33.25			
		32.87	37.74	30.02			
		33.18	36.87	34.19			
Level 3	111.82	111.87	116.31	103.17	111.41	10.51	9.43
		113.62	126.14	102.16			
		117.19	136.16	100.34			
		109.73	113.85	95.60			

		114.57	109.29	101.16			
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Cystine	Theoretical value	Measurement value			Average	SD	CV %
Level 1	3.11	3.34	2.99	2.93	2.97	0.14	4.57
		2.99	2.94	2.74			
		3.12	2.91	2.84			
		3.04	2.96	2.85			
		3.01	2.95	3.00			
Level 2	35.24	37.32	35.53	35.33	36.00	0.99	2.75
		37.46	34.84	36.60			
		37.38	34.91	35.82			
		34.59	35.33	36.53			
		36.34	35.11	36.85			
Level 3	114.00	121.78	111.63	116.33	117.16	3.63	3.10
		122.54	114.76	116.73			
		115.80	116.51	114.69			
		119.63	116.02	114.98			
		125.44	115.86	114.64			

[0082] LLMI

Analyte name	Citric acid	Oxalic acid	Cystine
Theoretical value	1.60	1.00	1.00
Measurement value	1.76	1.06	1.02
	1.69	1.04	1.03
	1.63	1.09	1.03
	1.69	1.05	1.04
	1.65	1.10	1.07
Average	1.68	1.07	1.04
SD	0.05	0.03	0.02
CV %	2.96	2.42	1.85
Bias %	5.25	6.80	3.80

[0083] 2.4 Linearity

Citric acid	SD1	SD2	SD3	SD4	SD5	SD6	R
Theoretical value	1.60	8.00	16.00	80.00	160.00	320.00	/

Measurement result	1.62	7.33	16.48	81.27	162.93	322.11	0.9990
	1.62	7.53	15.84	78.93	172.50	317.27	0.9988
	1.59	8.46	15.21	80.65	162.70	311.58	0.9992
Average	1.61	7.77	15.84	80.28	166.04	316.99	/
Bias %	0.63	-2.83	-0.98	0.35	3.78	-0.94	/

Oxalic acid	SD1	SD2	SD3	SD4	SD5	SD6	R
Theoretical value	1.00	5.00	10.00	50.00	100.00	200.00	/
Measurement result	0.99	5.05	10.98	49.09	99.05	186.20	0.9983
	1.01	4.77	10.31	48.35	102.90	202.34	0.9994
	1.00	4.78	10.47	50.31	101.22	194.82	0.9994
Average	1.00	4.87	10.59	49.25	101.06	194.45	/
Bias %	0.00	-2.67	5.87	-1.50	1.06	-2.77	/

Cystine	SD1	SD2	SD3	SD4	SD5	SD6	R
Theoretical value	1.00	5.00	10.00	50.00	100.00	200.00	/
Measurement result	1.01	4.83	10.06	49.22	101.57	204.38	0.9998
	1.00	4.93	9.95	48.24	101.04	208.07	0.9996
	1.01	4.72	9.95	50.19	103.71	201.85	0.9994
Average	1.01	4.83	9.99	49.22	102.11	204.77	/
Bias %	0.67	-3.47	-0.13	-1.57	2.11	2.38	/

[0084] Conclusion: all verification indexes are qualified.

[0085] Example 3

[0086] Stabilities of samples after addition of different preservatives at room temperature were investigated.

[0087] 1: no addition, 1HCl: add HCl, 1TL: add toluene, 1DU: add diazoalkyl ureas; others, and so on.

Citric acid	0 h	3 h	6 h	1 day	2 day	10 day	2 weeks
1	100.00	92.06	97.28	92.79	99.69	20.58	13.19
1 HCl	100.00	101.87	100.56	99.56	92.61	97.74	100.13
1 TL	100.00	106.33	107.49	101.22	106.10	95.65	97.55

1 DU	100.00	104.68	103.59	105.21	100.82	102.22	103.96
2	100.00	106.89	103.92	105.17	102.91	99.07	104.48
2 HCl	100.00	104.76	102.52	100.11	108.08	93.89	103.03
2 TL	100.00	98.92	101.13	102.54	91.93	98.37	99.52
2 DU	100.00	95.32	94.47	96.73	105.67	100.10	95.45
3	100.00	101.82	100.36	98.78	97.21	50.92	1.27
3 HCl	100.00	107.74	99.93	110.01	102.42	108.47	107.93
3 TL	100.00	98.46	98.90	98.56	97.33	68.95	3.96
3 DU	100.00	94.54	94.04	95.09	96.77	104.45	115.85

Oxalic acid	0 h	3 h	6 h	1 day	2 day	10 day	2 weeks
1	100.00	102.24	106.57	101.19	101.49	100.60	89.55
1 HCl	100.00	107.15	101.87	100.00	101.09	109.80	106.69
1 TL	100.00	111.57	105.18	105.94	104.57	107.15	103.04
1 DU	100.00	108.49	107.90	106.41	99.70	109.84	109.54
2	100.00	134.65	152.45	173.54	186.25	211.96	203.01
2 HCl	100.00	99.66	101.57	103.59	105.16	113.68	112.33
2 TL	100.00	114.00	139.76	168.33	186.75	209.96	203.76
2 DU	100.00	121.79	144.48	168.37	185.23	202.10	194.35
3	100.00	106.42	104.99	104.43	105.27	109.03	107.09
3 HCl	100.00	110.41	107.08	106.30	111.95	117.20	117.66
3 TL	100.00	102.55	103.41	102.72	106.39	106.57	106.69
3 DU	100.00	97.69	98.92	99.85	102.00	98.48	100.92

Cystine	0 h	3 h	6 h	1 day	2 day	10 day	2 weeks
1	100.00	105.21	109.24	110.66	118.72	121.56	104.27
1 HCl	100.00	105.58	111.93	105.33	107.61	137.31	117.26
1 TL	100.00	117.59	122.36	110.80	125.88	163.82	138.69
1 DU	100.00	119.68	111.97	104.52	101.33	123.40	111.44
2	100.00	101.21	106.79	102.56	102.11	90.80	78.58
2 HCl	100.00	110.02	115.53	109.18	115.36	132.05	132.22
2 TL	100.00	110.19	112.35	111.57	114.66	111.73	101.70
2 DU	100.00	109.85	111.38	108.83	103.57	119.69	110.53
3	100.00	116.40	126.44	118.22	124.58	150.87	154.98
3 HCl	100.00	113.76	118.44	109.74	116.15	123.09	122.47
3 TL	100.00	108.63	112.94	113.99	112.71	145.89	144.76
3 DU	100.00	106.54	107.06	95.56	99.51	109.51	105.84

[0088] It can be seen through the above results that 24 h urine is acidified by adding hydrochloric acid before samples are collected, and oxalic acid and citric acid in samples are stably placed for 2 weeks at room temperature.

[0089] Although the present disclosure has been described in detail with general description and specific embodiments above, it is obvious for those skilled in the art that some modifications or improvements can be made on the basis of the present disclosure. Therefore, these modifications or improvements made without departing from the spirit of the present disclosure belong to the scope of the present disclosure.

Claims

1. A method for simultaneously detecting oxalic acid, citric acid and cystine in 24h urine, comprising the following steps:

S1, preparation of a test sample: taking a to-be-detected 24h urine sample, and adding oxalic acid-1,2-¹³C₂ as an internal standard of oxalic acid, citric acid-2,2,4,4-d₄ as an internal standard of citric acid and L-cystine-d₄ as an internal standard of cystine; and performing derivation treatment followed by successively adding a tetramethyl ammonium fluoride solution and an acetonitrile solution to be evenly mixed, then injecting the mixed solution onto a weak cation exchange 96-well plate, and collecting eluent as the test sample; and

S2, detection: detecting the test sample utilizing a liquid chromatography tandem mass spectrometer.

2. The method for simultaneously detecting oxalic acid, citric acid and cystine in 24h urine according to claim 1, wherein in step S1, the derivation treatment comprises the following steps: successively adding benzyl alcohol and trimethylchlorosilane into the 24h to-be-detected urine sample added with an internal standard working solution to be evenly mixed by vortex vibration, and then putting the obtained mixture in an oven to be derived at 80°C.

3. The method for simultaneously detecting oxalic acid, citric acid and cystine in 24h urine according to claim 1, wherein in the internal standard solution, the concentration of oxalic acid-1,2-¹³C₂ is 25 µg/mL, the concentration of citric acid-2,2,4,4-d₄ is 10 µg/mL, and the concentration of L-cystine-d₄ is 12.5 µg/mL.

4. The method for simultaneously detecting oxalic acid, citric acid and cystine in 24h urine according to claim 1, wherein in step S2, the detection conditions of liquid chromatography are as follows:

mobile phase A of a high-pressure pump of a liquid chromatography system: 0.1%

formic acid-2 mM ammonium acetate-aqueous solution;

mobile phase B of a high-pressure pump of a liquid chromatography system: 0.1%

formic acid-2 mM ammonium acetate-methanol solution;

a gradient elution program is as follows:

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0.00	60	40
0.50	60	40
1.00	25	75
2.60	25	75
2.70	0	100
3.40	0	100
3.50	60	40
4.00	60	40

5. The method for simultaneously detecting oxalic acid, citric acid and cystine in 24h urine according to claim 4, wherein in chromatographic conditions, a chromatographic column is henomenex F5 Column, 2.6 μ m, 100A, 3.0×50 mm, and the flow rate of the mobile phase is 0.6 mL/min.

6. The method for simultaneously detecting oxalic acid, citric acid and cystine in 24h urine according to claim 4, wherein in chromatographic conditions, the column temperature is 40°C, and the injection amount is 5 μ L.

7. The method for simultaneously detecting oxalic acid, citric acid and cystine in 24h urine according to claim 1, wherein in step S2, mass spectrum conditions are as follows: an electrospray ionization source, a positive ion mode, spraying voltage: 5500V; temperature: 500°C; atomization air: 55 psi; auxiliary heating air: 55 psi; air curtain: 25 psi; CAD: 9; specific monitoring ion pairs and collision energy are as follows:

Analyte	Q1Mass	Q3Mass	Time (msec)	DP	CE	CXP	Corresponding internal standard
Dibenzyl Oxa-1	288.1	108.1	30	82	13	12	Dibenzyl Oxa-13C2-1
Dibenzyl Oxa-2	288.1	181.0	15	82	11	11	Dibenzyl Oxa-13C2-2
Tribenzyl Cit-1	463.1	237.1	30	75	12	7	Tribenzyl Cit-d4-1

Tribenzyl Cit-2	463.1	283.1	15	75	10	9	Tribenzyl Cit-d4-2
Dibenzyl Cys-1	421.1	210.1	30	110	27	12	Dibenzyl Cys-d4-1
Dibenzyl Cys-2	421.1	300.1	15	110	24	9	Dibenzyl Cys-d4-2
Dibenzyl Oxa-13C2-1	290.1	108.1	30	80	13	12	
Dibenzyl Oxa-13C2-2	290.1	181.0	15	80	12	11	
Tribenzyl Cit-d4-1	467.2	241.1	30	75	12	7	
Tribenzyl Cit-d4-2	467.2	287.1	15	75	10	9	
Dibenzyl Cys-d4-1	425.2	212.1	30	110	27	12	
Dibenzyl Cys-d4-2	425.2	302.1	15	110	24	9	

8. The method for simultaneously detecting oxalic acid, citric acid and cystine in 24h urine according to claim 1, also comprising: using stable isotope labeled oxalic acid-1,2-¹³C₂ as an internal standard of oxalic acid, citric acid-2,2,4,4-d₄ as an internal standard of citric acid and L-cystine-d₄ as an internal standard of cystine; plotting a standard curve by preparing a series of standard products with different concentrations, performing linear regression analysis by using the concentration of a standard solution as X axis and using a ratio of standard and internal standard peak areas as Y axis, and obtaining a regression equation via “1/X” weight; and substituting a ratio of a peak area of a to-be-detected component in the sample to an peak area of an internal standard of the component into the standard curve equation to calculate concentrations of to-be-detected components in the urine sample.

9. The method for simultaneously detecting oxalic acid, citric acid and cystine in 24h urine according to claim 1, also comprising: measuring background values of oxalic acid, citric acid and cystine in an artificial matrix, then adding known concentrations of oxalic acid, citric acid and cystine to the artificial matrix, detecting by a liquid chromatography tandem mass spectrometer, and comparing actually measured oxalic acid, citric acid and cystine with theoretically calculated oxalic acid, citric acid and cystine to judge the reliability of the liquid chromatography tandem mass spectrometer.

10. Use of a kit in urine metabolism assessment of a nephrolithiasis patient,

wherein the kit comprises a marker composition consisting of oxalic acid, citric acid and cystine, and the marker composition is detected by using the method for simultaneously detecting oxalic acid, citric acid and cystine in 24h urine according to any one of claims 1 to 9.