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Development and validation of a simple determination of urine metabolites (oxalate, citrate, uric acid and creatinine) by capillary zone electrophoresis

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ABSTRACT

Oxalate, citrate, uric acid and creatinine are important urine markers for the evaluation and treatment of urolithiasic patients. They have been traditionally analysed by enzymatic and chromatographic techniques which present practical drawbacks, mainly in the sample pre-treatment step. The purpose of this study was to evaluate those markers in urine samples, by an easy multi-analyte assay using capillary zone electrophoresis. The four urine metabolites were determined, at 25 °C, by using a 50 cm × 75 μm capillary in 50 mmol l^{-1} phosphate buffer (pH 6.5), at constant voltage of −30 kV and UV detection at 195 nm (for oxalate and citrate) or 30 kV and 234 nm (for creatinine and uric acid). The sample pre-treatment was minimum, 5- and 20-fold dilution of the urine sample and acidification to pH 3–4. Validation parameters (linear range, sensitivity, accuracy, precision and detection limits) were statistically comparable to those obtained with the official methods normally used in the clinical practice. The effect of freezing as a conservation method of urine samples is also discussed in terms of recoveries of the analytes. The analytical method developed is highly useful as a diagnostic tool for detecting metabolic renal disorders due to its simplicity, time consuming, easy automation, cost efficiency and analytical effectiveness, accomplishing with the clinical requirements.

1. Introduction

Urolithiasis is a common disease, affecting 12% of the European Union population along their life [1,2] and characterized by a high prevalence and incidence, high morbidity and high rates of recurrence. The recurrence rate during 5 years lies between 44 and 75% [3], either if the lithiasic episode has been solved spontaneously or by urologic intervention, due to the persistence of the urinary alteration which causes the renal stone formation. Such alteration may be diagnosed and treated by dietetic changes [4], pharmacological treatment, or both, decreasing the aggressiveness of the disease and, consequently, the number of recurrences [5].

The chemical composition of the majority of stones is calcium oxalate (70%) being the rest calcium phosphate (hydroxyapatite or brushite), magnesium and ammonium phosphate (struvite), uric acid and cystine principally [6,7].

A lithiasic episode begins when the equilibrium between supersaturation, promoters, inhibitors and morphoanatomic factors is broken, being the main phenomena the supersaturation of several compounds in urine that might crystallise forming solid concretions, influenced by the scarcity of crystallisation inhibitors, the presence of crystallisation promoters and morphoanatomic factors [8]. It takes place, for example, in those cases with high concentration of lithogenic compounds (hypercalciuria, hyperoxaluria, hyperuricuria, hyperphosphaturia...) or those with low concentration of inhibitors (hypomagnesiuria, hypocitraturia...).

Accurate evaluation of alterations and its magnitude in each individual patient are required to develop efficient therapeutic treatments and to diagnose the risk of lithiasic disease in healthy people, with the consequent advantage in the prevention of the disease. The determination of the urinary levels of calcium, magnesium, phosphorus, oxalate, citrate and uric acid has long been recognized as an important factor in the evaluation and treatment of urolithiasic patients. Another interesting analyte is creatinine, which serves as one of the most widely used markers of renal function (indicator of the glomerular filtration rate of kidneys what is the amount of fluid filtered per unit time). While calcium, magnesium and phosphorus are easily measured by atomic spectroscopy, the measurement of oxalic acid, citric acid, uric acid and creatinine involve some practical drawbacks which have been exhaustively reviewed [9]. For these compounds, enzymatic techniques are widely used (oxalate [10,11], citrate [12], uric acid [13] and creatinine [14]) which are usually characterized by consumption of large amounts of reagents, inability to test multiple analytes at a time and laborious sample treatment, requiring dilution, filtration, centrifugation, decolourisation, pH adjusting and need of incubation, with the consequent increase

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of time for analysis, and lack of specificity for the target analyte (i.e. uricase method is interfered by guanine, xanthine and a few other structural analogs of uric acid [13]). Ion chromatography or high-performance liquid chromatography have also been applied for determining oxalate [15,16], citrate [16,17], uric acid [18] and creatinine [18] which are characterized by consumption of large amounts of reagents, need of sample pre-treatment (with the possibility of losing analyte and/or introduce contamination) and requirement of different kind of columns for the analysis of the four proposed analytes. Gas chromatography with mass detection methods for oxalate analysis has also been proposed providing structural information but requiring relatively clean samples [19]. Many other methods such as the phosphotungstic acid method, mainly interfered by proteins, glucose, caffeine, ascorbic acid... present in urine [13], electrochemical techniques for uric acid [20] or the Jaffé's reaction for creatinine, interfered by several substances in urine such as proteins, ascorbic acid, guanidine... [13,21] also present several drawbacks such as consumption of large amounts of reagents, inability to test multiple analytes at a time or lack of specificity for the target analyte.

Nowadays capillary zone electrophoresis (CZE) has become an efficient analytical separation technique with an increasing impact in the clinical laboratory [22,23]. Preliminary attempts in determining separately oxalate and citrate [24,25], and uric acid and creatinine [26,27] by capillary electrophoresis have been reported. CZE has several advantages including the need of minimal amounts of sample and solvent consumption, low costs of analysis and simple sample treatment. Additional advantages of the technique are multicomponent analysis capabilities, high specificity, sensitivity and resolution, good reproducibility, high sample throughput and easy automation.

The main objective of the present study is the validation of a method for direct measurement of oxalate, citrate, uric acid and creatinine in urine by using capillary zone electrophoresis. The influence of different operational parameters such as the concentration and pH of running buffer and applied voltage, on the resulting separation have been investigated. The method has been validated and successfully applied to urine samples demonstrating its easy application and usefulness.

2. Materials and methods

2.1. Apparatus

CZE was performed on a Beckman P/ACE MDQ (Beckman Instruments, Palo Alto, CA, USA) equipped with a PDA (Photodiode Array) detector set at 195 and 234 nm. Data collection and analysis were carried out by Beckman P/ACE System MDQ software.

Separations were performed on a polyimide-coated fused-silica capillary of 50 cm length, with an internal diameter of 75 μm. The detector window was set at 40 cm from the inlet.

When new capillaries were used, were previously washed during 60 min with 1 mol l^{-1} NaOH followed by 10 min of deionised water at 20 psi. Each day, the capillary was rinsed for 20 min with 0.1 mol l^{-1} NaOH, 2 min with deionised water, and 10 min with the related run buffer solution at 20 psi. Before each run, the capillary was rinsed for 1 min with 0.1 mol l^{-1} NaOH, 1 min with deionised water, and 2 min with the run buffer solution at 20 psi.

All experiments were carried out at 25 °C. Sample injections were made under pressure of 0.5 psi during 5 s and by triplicate. Whereas injection was made in the cathode and detection in the anode for oxalate and citrate with a separation voltage set at −30 kV, injection was made in the anode and detection in the cathode for creatinine and uric acid by reversing the applied voltage to +30 kV.

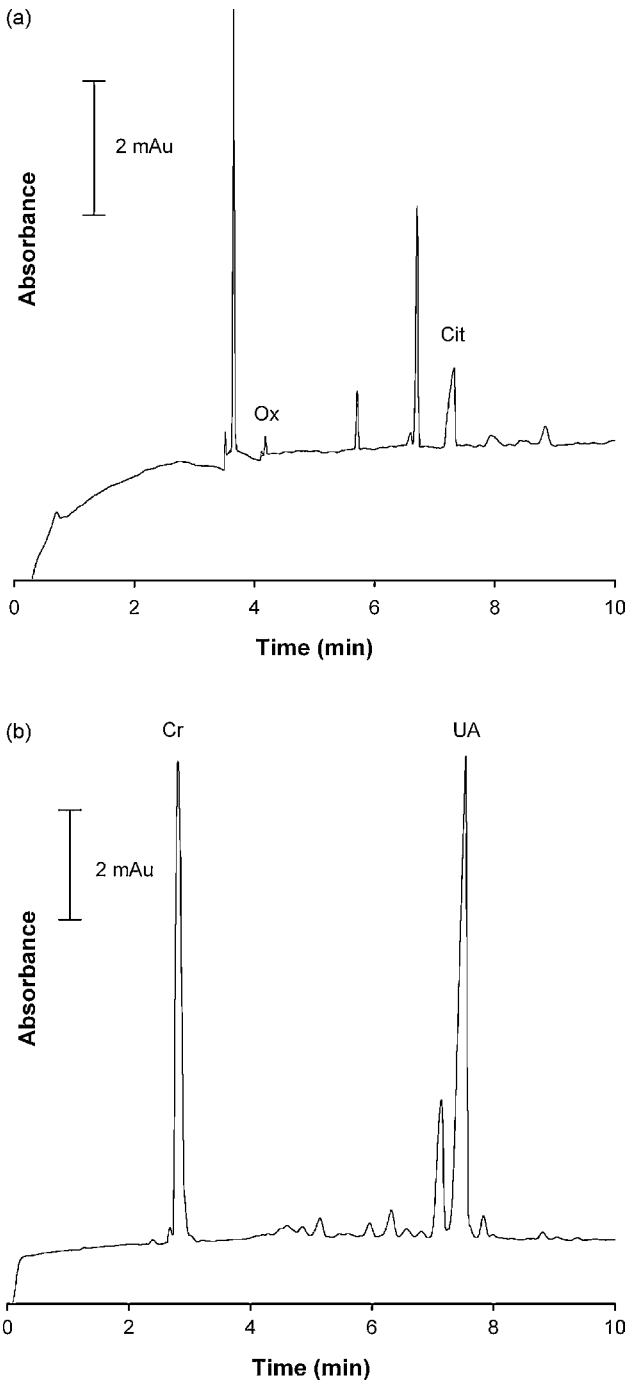

Fig. 1. (a) Electropherogram of a 5-fold diluted urine sample. Separation performed at −30 kV in 50 mmol l^{-1}, phosphate buffer (pH 6.5), detection at 195 nm. Ox: oxalate; Cit: citrate. (b) Electropherogram of a 20-fold diluted urine sample. Separation performed at +30 kV in 50 mmol l^{-1}, phosphate buffer (pH 6.5), detection at 234 nm. Cr: creatinine; UA: uric acid. Identification of the analytes peaks was carried out by spiking urine samples with an additional amount of the analyte to be tested.

2.2. Reagents

All reagents used were analytical grade. Sodium oxalate and uric acid were from Panreac (Barcelona, Spain). Sodium citrate was from Merck (Darmstadt, Germany). Creatinine was from Aldrich (Milwaukee, WI, USA). Potassium dihydrogen phosphate (KH_2PO_4) was from Merck (Darmstadt, Germany) and sodium hydroxide (NaOH) from Panreac (Barcelona, Spain). Standards, buffer solutions and all dilutions were prepared with milli-Q water.

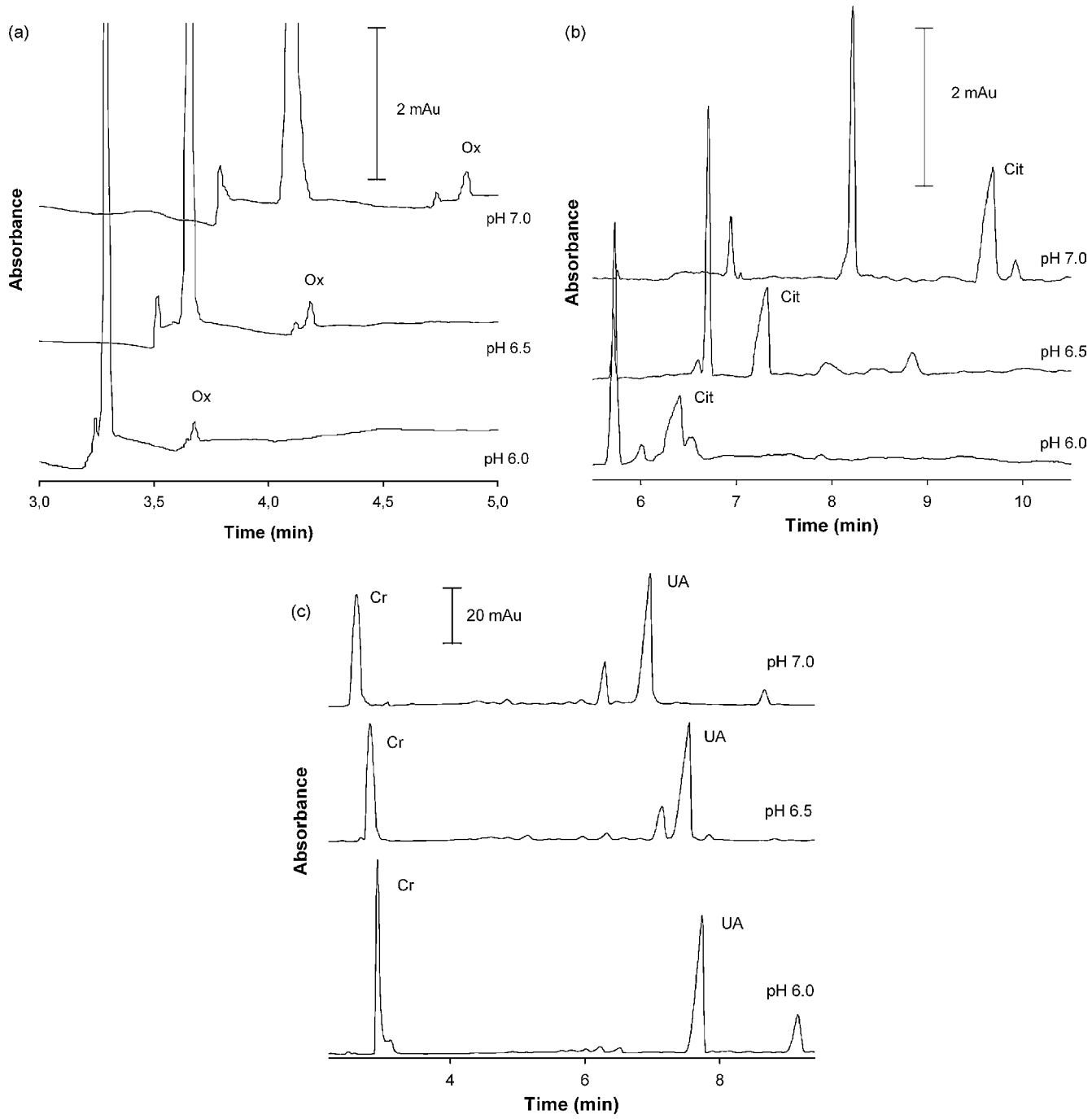

Fig. 2. (a) Effect of pH on oxalate separation. (b) Effect of pH on citrate separation. (c) Effect of pH on creatinine and uric acid separation. Conditions as stated in Fig. 1.

2.3. Electrophoretic buffer

The electrophoretic buffer was prepared by 10-fold dilution of KH_2PO_4 500 mmol l^{-1} and adjustment to pH 6.5 with NaOH 1.0 mol l^{-1}. Fresh buffer was daily prepared.

2.4. Samples, sample pre-treatment and sample stability

Urine samples used for optimisation and validation of the proposed method were kindly provided by the members of our research group. They were analysed before and after storage at −20 °C. A minimum sample pre-treatment was carried out as follows: prior to analysis, samples were gently shaken and diluted 1:5 with HCl 40 mmol l^{-1} (final pH 2–3) for oxalate and citrate analysis and 1:20 with HCl 40 mmol l^{-1} (final pH 2–3) for creatinine and uric acid analysis and filtered through 0.22 μm pore-size filters before injection in the CE equipment.

2.5. Comparison with official methods

Six urine samples were analysed in the clinical laboratory of the Hospital Universitari de Bellvitge following the official methods: oxidase–peroxidase method for oxalate (Trinity Biotech, ref. 591-C), lyase–dehydrogenase method for citrate (Roche Diagnostics, ref. 139,076), uricase–peroxidase method for uric acid (Roche Diagnostics) and Jaffé's method for creatinine (Roche Diagnostics). Results of these analyses were statistically compared with those obtained by applying the proposed CZE procedure.

2.6. Validation of method and Statistical analysis

Validation parameters as linear range, sensitivity, accuracy, precision and detection and quantification limits were determined. Mean values of samples and their standard deviation (the target CV for all measurements was <10%) were calculated and the paired Student's *t*-test ($P < 95\%$) was performed for statistical means comparison. ANOVA analysis was carried out in order to compare results obtained for the target analytes at different conditions.

3. Results and discussion

Preliminary attempts of determining the four substances in a 1:5 diluted sample in a single run by using phosphate buffers (25–100 mM) with cetyltrimethylammonium bromide (0.05–1.0 mM) for reversing the electroosmotic flow at pH 5.5–7.5 and −10 kV to −30 kV resulted in overlapped analyte peaks with some matrix components and/or saturation of photometric signal for uric acid and creatinine. For 1:10 or 1:20 diluted samples oxalate and citrate resulted in an insufficient sensitivity for its accurate determination. So, the determination of oxalate, citrate, uric acid and creatinine in urine can not be accomplished by a direct run by using UV photometric detection due to the complexity of urine matrix, the different concentration levels of the analytes in the urine and the different sensitivity of CE for the analytes of interest. Then this determination is best carried out by processing two aliquots of the urine sample, i.e. a low-diluted sample for oxalate and citrate analysis (low sensitivity of detection) and a high-diluted sample for uric acid and creatinine analysis (high sensitivity of detection) and using the same buffer with a different voltage polarity for each dilution condition. Thus, oxalate and citrate are determined by applying a voltage of −30 kV with a 5-fold dilution (Fig. 1a), whereas uric acid and creatinine are determined in the same buffer by applying a voltage of +30 kV and 20-fold dilution (Fig. 1b).

3.1. Method optimisation: study of variables

Phosphate buffers are usually used when direct UV/vis absorbance detection is used. Preliminary results showed a KH_2PO_4 50 mmol l^{-1} to be adequate for the proposed separation scheme, as a result of a compromise, i.e., lower concentrations (10–25 mmol l^{-1}) of the buffer resulted in an insufficient buffering for the injected urine samples leading to broadened peaks, whereas higher concentrations (100 mmol l^{-1}) produced high current through the capillary causing degradation of the separation and non-reproducible migration times. The pH of the buffer is the most critical parameter on altering the selectivity of the capillary between solutes in a CZE separation. At pH 6.0, oxalate and citrate resulted in overlapped peaks, without baseline resolution (Fig. 2a and b). When pH increases up to 7.0, the resolution between oxalate and citrate and their interferences increases, resulting pH = 6.5 the optimum, offering enough resolution for both analytes and shorter extent of the analysis. Creatinine and uric acid were well-resolved for the three pH conditions assayed (Fig. 2c). Thus, pH 6.5 was selected as the optimum pH suitable for both determinations: oxalate and citrate, and creatinine and uric with the same buffer by simply reversing the polarity of the electrodes between the two runs. Applied voltage was set to −30 kV and +30 kV, respectively, since lower voltages (20 and 25 kV) not only did not improve the separation but did extent considerably the analysis time.

3.2. Validation

Main validation parameters results for oxalate, citrate, uric acid and creatinine are shown in Table 1. As shown there, standards fit the linear model ($R > 0.999$) for all the analytes in the indicated ranges and no matrix effects were found for any of the analytes, since there are no statistically significant differences (at 95% confidence level) in the slopes from direct calibration and those from standard additions.

Table 1
Main validation parameters of CE method (a: intercept; b: slope; C.L.: confidence limits: 95%).

	R		*a* ± C.L.		*b* ± C.L.		Range (mg l^{-1})	Sample dilution
	Standards linearity	Standard addition	Standards linearity	Standard addition	Standards linearity	Standard addition		
Oxalate	0.9991	0.9999	0 ± 15	140 ± 6	29 ± 2	28.1 ± 0.9	2–10	1:5
Citrate	0.9999	0.9973	−19 ± 20	1090 ± 60	10.3 ± 0.2	10.0 ± 1.0	40–200	1:5
Uric acid	0.9997	0.9995	300 ± 700	10,500 ± 700	173 ± 7	166 ± 6	10–50	1:20
Creatinine	1.0000	0.9999	300 ± 500	34,900 ± 600	237 ± 7	239 ± 5	20–100	1:20

	Limit of detection, LoD (mg l^{-1})	Limit of quantification, LoQ (mg l^{-1})
Oxalate	0.46	1.5
Citrate	2.6	8.7
Uric acid	3.8	13
Creatinine	1.3	4.3

	Mean recovery (%)	R.S.D. (%)	Range (%)
Sample accuracy ($n = 13$)			
Oxalate	101.0	2.6	96.3–104.7
Citrate	99.6	2.6	96.7–103.9
Uric acid	99.3	3.1	96.3–104.2
Creatinine	100.5	2.9	96.1–104.0

	Migration time (min)		Migration time (% R.S.D.)		Area (% R.S.D.)	
	Standards precision ($n = 10$)	Sample precision ($n = 10$)	Standards precision ($n = 10$)	Sample precision ($n = 10$)	Standards precision ($n = 10$)	Sample precision ($n = 10$)
Oxalate	3.98	4.14	2.0	2.3	2.7	3.2
Citrate	7.13	7.33	2.2	2.8	1.8	2.4
Uric acid	7.62	7.55	1.7	2.4	1.6	2.7
Creatinine	2.73	2.80	1.1	1.6	1.2	1.5

Table 2
Measurements of oxalate, citrate, uric acid and creatinine in 13 samples stored under different conditions: fresh (analysed immediately after collection), stored at room temperature during 24 h and after freezing during 1 month at −20 °C.

Sample	Oxalate (mg l^{-1})			Citrate (mg l^{-1})			Creatinine (mg l^{-1})			Uric Acid (mg l^{-1})		
	Fresh	24 h	Frozen	Fresh	24 h	Frozen	Fresh	24 h	Frozen	Fresh	24 h	Frozen
1	22.5	23.2	22.7	755.5	831.1	791.6	2264.6	2249.5	2242.9	808.7	834.6	842.3
2	30.2	31.1	31.5	461.4	447.2	471.4	2237.3	2220.7	2309.4	928.3	989.8	997.0
3	22.2	22.7	22.0	433.1	445.5	428.4	1753.1	1794.6	1756.6	937.4	939.5	873.1
4	8.4	11.2	11.4	141.4	196.3	155.1	676.5	698.1	719.1	350.3	367.6	350.2
5	14.6	12.7	14.2	384.9	382.5	348.1	1511.4	1475.9	1441.0	809.3	748.9	759.5
6	21.7	24.4	21.4	237.4	284.8	254.5	1328.1	1309.3	1315.4	828.0	798.4	789.6
7	55.0	55.7	55.6	937.6	930.3	942.0	994.7	941.2	1016.4	693.1	611.7	647.6
8	25.5	30.6	31.4	863.7	846.3	811.6	1667.5	1519.1	1557.0	1054.9	995.7	1030.3
9	6.5	2.6	4.6	317.0	307.3	320.2	540.3	412.0	450.6	257.2	269.1	231.2
10	5.4	8.9	7.7	222.8	180.8	194.8	178.9	206.1	187.8	77.7	97.6	95.0
11	8.4	8.1	8.0	368.4	418.6	388.1	214.4	174.6	141.6	134.6	119.4	131.9
12	30.3	27.0	29.9	648.3	689.8	644.8	1365.0	1185.7	1220.8	542.7	557.6	525.5
13	16.9	14.8	15.6	671.0	606.1	635.9	1123.7	1092.2	1030.0	560.6	535.1	518.7

The limits of detection (LoD) were determined as three times the standard deviation, $s_{y/x}$, of the residuals of the calibration linear model, divided by the sensitivity (slope) for a given analyte [28]. Similarly, the limits of quantification (LoQ) were calculated as ten times $s_{y/x}$ divided by the sensitivity for a given analyte. LoD and LoQ for the analytes of interest are given in Table 1. Detection limits provided for the present method are under the lowest values found either in the randomly assayed samples or in the practice in the clinical laboratory. The linear ranges and limits of quantification are adequate to cover all urinary concentrations of the analysed markers in human urine, after corresponding dilution (5-fold dilution for oxalate and citrate and 20-fold dilution for uric acid and creatinine).

The accuracy of the method was determined by the evaluation of the recoveries for the four analytes from 13 urine samples analysed by standard addition. Recoveries were near 100%, ranging from 96 to 105% (Table 1). Due to the possibility of calcium oxalate and calcium citrate complex formation during urine analysis, which would produce a negative interference, the effect of calcium concentration and pH of the samples was also studied found to be independent from calcium content in urine and pH of samples (data not shown), accordingly with the adequate selectivity of the analytical method for the four analytes of interest. From these results it can be concluded that calcium and pH, into their physiological urinary range, do not interfere with the oxalate and citrate determination. Obviously, any crystallisation reaction previous to the analysis, i.e. during sample storage, can produce a negative error. Thus, it is highly important to assure the total dissolution of the sample (by the combined action of dilution and acidification) prior to the analysis.

Executing 10 runs of the same standard and sample (at the middle concentration of the calibration curves), migration times showed R.S.D.s under 3.0% (Table 1), repeatability low enough to consider the method acceptable, since peak assignment is performed mainly with this criterion. Peak areas showed R.S.D.s lower than 3.2% (Table 1), again low enough to perform adequate quantitative analysis.

If a 100% recovery for all the samples was achieved, a linear relationship, $Y = X$, should be obtained when plotting the analyte concentration determined in natural samples, Y, versus the analyte concentration found in the same natural sample after spiking it and correcting the result by the spiked amount, X. Results obtained fit to linear equations, by the least-square method, which are statically comparable at a 95% confidence level to the graph $Y = X$ for oxalate [$Y = -0.3(\pm 0.6) + 1.00(\pm 0.03)X$, $R = 0.9993$], citrate [$Y = 2 \pm (15) + 1.00(\pm 0.03)X$, $R = 0.9992$], uric acid [$Y = 14(\pm 15) + 0.98(\pm 0.04)X$, $R = 0.9994$] and creatinine [$Y = -1 \pm (30) + 1.00(\pm 0.02)X$, $R = 0.9995$], each of them with $n = 13$.

Samples were frozen at −20 °C after the first analysis and measured again 1 month later to assess stability of the compounds under freezing conditions. Moreover, fresh samples (acidified and diluted aliquots) were reanalysed after 24 h of the first analysis to assess the stability of the analytes at room temperature. The results were subjected to ANOVA analysis, at a 95% confidence level, showing no significant differences in any of the target analytes for any of the mentioned conditions (Table 2). Thus, urine diluted and acidified samples are stable during a minimum of 24 h at room temperature for the analysis of oxalate, citrate, creatinine and uric acid. In case that the analysis was not able to be performed in the next 24 h after collection, freezing at −20 °C assures the stability of the analytes of interest during at least 1 month.

Finally, a comparison of the CZE measurement of the four urinary markers with the corresponding official methods is shown in Table 3. The paired Student t-test indicated that there is no statistical difference ($P < 95\%$) between the results obtained by both methods for the assayed metabolites.

Table 3
Measurements of oxalate, citrate, uric acid and creatinine by CZE and official methods in six urine samples[a].

Sample	Oxalate (mg l^{-1})		Citrate (mg l^{-1})		Uric acid (mg l^{-1})		Creatinine (mg l^{-1})	
	CZE	Official	CZE	Official	CZE	Official	CZE	Official
1	16.0	19.7	1574	1223	607	512	1566	1599
2	11.3	16.3	1041	1143	545	497	1982	2051
3	7.6	7.1	176	215	262	218	482	469
4	20.1	19.0	882	662	528	553	1687	1655
5	15.8	12.3	313	246	693	585	1084	1109
6	21.4	17.3	517	548	482	538	1783	1834

[a] Paired t-test: Oxalate, $t_{cal} = 0.042$, $t_{tabl} = 2.571$; Citrate, $t_{cal} = 1.089$, $t_{tabl} = 2.571$; Uric acid, $t_{cal} = 1.359$, $t_{tabl} = 2.571$; Creatinine, $t_{cal} = 1.416$, $t_{tabl} = 2.571$.

4. Conclusion

A method has been validated for measuring oxalate, citrate, uric acid and creatinine in urine samples by CZE. Accordingly with their concentration levels and sensitivity of detection, two different dilution factors are used for the determination of oxalate and citrate (1:5) and creatinine and uric acid (1:20) by applying a corresponding reverse potential to each of the diluted urine samples.

The CE analysis offers many advantages to current testing methods, including simplicity, easy of automation, high throughput (time of analysis < 20 min), high specificity, minimal sample volume requirements, reduced cost of capillary and electrolyte materials, minimal sample preparation (requiring only dilution–acidification and filtration of the urine samples), the ability to simultaneously analyse multiple markers, accuracy and reproducibility. A further potential advantage of CE is that it can also be used to measure other important urinary components.

The simplicity of the whole process, the demonstrated good performance and high throughput (50 samples per day) make it recommendable in routine clinical analysis.

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