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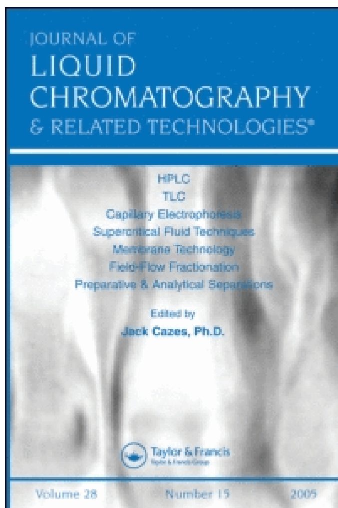

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DIRECT SIMULTANEOUS DETERMINATION OF UREMIC TOXINS: CREATINE, CREATININE, URIC ACID, AND XANTHINE IN HUMAN BIOFLUIDS BY HPLC

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DIRECT SIMULTANEOUS DETERMINATION OF UREMIC TOXINS: CREATINE, CREATININE, URIC ACID, AND XANTHINE IN HUMAN BIOFLUIDS BY HPLC

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ABSTRACT

The determination of creatine, creatinine, uric acid, and xanthine in urine and blood serum is very important in clinical assays as they are most widely used as markers to assess renal function.

A simple and direct method for the routine analysis of uremic toxins: creatine, creatinine, uric acid, and xanthine in human blood serum and urine is described. Low wavelength UV detection is achieved at 200 nm using 10 mmol/L KH_2PO_4, as mobile phase, at a flow rate 0.8 mL/min and a Kromasil C_8, 250 × 4 mm, analytical column. Analysis was achieved within approximately 8 min.

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The limits of detection were 4 pg for creatine and creatinine, 20 pg for uric acid, and 6 pg for xanthine, while the limits of quantitation were 10 pg for creatine and creatinine, 60 pg for uric acid, and 20 pg for xanthine, when 20 μL were injected onto column. A rectilinear relationship was observed up to 2 ng/μL for creatine and creatinine, 12 ng/μL for uric acid, and 5 ng/μL for xanthine.

The statistical evaluation of the method was examined performing day-to-day ($n = 8$) and within-day ($n = 8$) calibration, and was found to be satisfactory with high accuracy and precision results. RSD values were in the range of 0.4 to 4.3% for within-day measurements and 3.5 to 7.4% for day-to-day precision measurements.

The developed method was applied to the analysis of creatinine, creatine, uric acid, and xanthine in biofluids: serum and urine, simply after dilution. The sensitivity of this method was high enough to determine the concentration of creatinine and uric acid in diluted serum (10 to 20 fold dilution) and urine (400–500 fold dilution) samples. Percentage recovery of analytes in spiked samples was in the range 91–105%. No interference was observed from endogenous compounds of human serum and urine. Correlation of analyzed samples using the developed method and conventional routine methods for creatinine and uric acid gave not significantly different results.

The method appears to be a very useful tool in routine analysis of clinical samples, for simultaneous determination of creatinine, creatine, uric acid, and xanthine levels in serum and urine.

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INTRODUCTION

Reliable determination of uremic toxins, creatine, creatinine, uric acid, in biofluids is still a matter of great importance in clinical chemistry. Their chemical structure is shown in Figure 1. Creatine is present in muscle, brain, and blood and although, not present in large amounts in normal urine from adults, it is abundantly present in the urine of adults who have recently ingested creatine supplements. Ingestion of a creatine supplement has been shown to increase the level of phosphocreatine, and this has become extremely popular in recent years with many athletes that want increased muscular power and performance enhancement by means of increase in muscle size and body mass. Because of this, analysis for creatine has become more important in the clinical setting.

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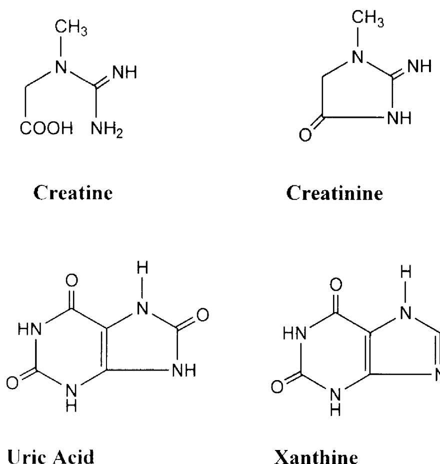

Figure 1. Chemical structures of examined compounds.

Additionally, elevated serum concentrations of creatine can be observed in some cases of muscle catabolism, such as primary myopathy, myositis, and muskelatrophie, as well as in the case hyperthyroidism. (1–4)

Creatinine is the end product of creatine catabolism. It results from the irreversible non-enzymatic dehydration and loss of phosphate from phospho-creatine. It is found in muscle tissue, blood, and urine. Creatinine concentrations are very useful indexes for evaluating glomerular filtration rate, and in general, is an important clinical marker of renal function. Urine creatinine is used to adjust the values of urinary biological indicators, e.g. creatinine measurements are necessary for correction of vanilmandelic (VMA) and homovanillic (HVA) acids, which indicate neuroblastoma and pheochromocytoma, when excreted in urine in abnormally increased concentrations. Ratios of VMA and HVA to creatinine have been utilized as a diagnostic index of these diseases. (2,5,6)

Oxypurines (uric acid and xanthine) are excreted in urine. Uric acid is a main end product of urine metabolism in the kidney. It is the final product of catabolization of the purine nucleosides. Uric acid, like creatinine, is also a marker for renal failure, as well as toxicity. Xanthines are intermediates of the metabolism of adenine and guanine to uric acid and, therefore, important analytes for diagnosis of certain types of metabolic disease. Uric acid and xanthines are markers for metabolic disorders, such as gout, Lesch-Nyman syndrome, and xanthinuria. Measurements of urinary excretion of purine metabolites, among them uric acid and xanthine, have been proposed as a marker for microbial protein synthesis. Their simultaneous determination is useful for diagnosis and treatment of hyperuricemia. (2,4,7–9)

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Obviously, the determination of such compounds is crucial for diagnosis and monitoring of renal disease and metabolic disorders.

Measurement of creatinine in serum and urine is routinely performed by photometric methods, such as the Jaffé reaction, while determination of uric acid is performed by enzymatic methods or, colorimetrically, by reduction of phosphotungstate. The colorimetric determination of creatinine by the Jaffé method is one widely accepted, and involves the formation of an adduct of creatinine with picric acid in alkaline solution, whose absorbance is measured at 500 nm. However, it is unspecific and subject to perturbation by many interfering substances from endogenous and exogenous origin. (6,10–12) Therefore, great efforts have been undertaken to improve existing methods or develop new measurement principles, using enzymatic and HPLC based methods. Chromatographic techniques are attractive for clinical analysis because of the inherent ability to analyze multiple component biofluids and determine the analytes of interest with minimal interference from other species. The chemical methods give higher values due to the presence of endogenous and exogenous substances. HPLC methods that can be found in literature involve ion-exchange, ion-pairing, reversed phase for the analysis of creatinine, and ion-exchange, ion-pairing, reversed phase, and size exclusion for uric acid. Most of these require deproteinization before analysis, but direct analysis of serum uric acid and creatinine was also successful by column switching liquid chromatography. Liquid-liquid extraction for isolation of creatinine among other compounds has been reported. Capillary electrophoretic methods are also reported for the determination of creatine and creatinine. (4,6,9,10,13–20)

Several papers can be found in literature dealing with the determination of single analytes or simultaneous determination of creatinine and uric acid, but there is no assay reported on the simultaneous determination of creatine, creatinine, uric acid, and xanthine in human biofluids.

The purpose of this report is to describe an HPLC method with UV detection that will permit the analysis of human serum and urine for the determination of creatinine, creatine, uric acid, and xanthine in a single run. This procedure is simple and rapid, and it does not require any sample pretreatment other than dilution and filtration. The procedure is proposed as an alternative to less specific colorimetric methods for routinely performed determination of examined compounds in clinical assays.

EXPERIMENTAL

Chemicals

Creatine hemisulfate salt, creatinine hydrochloride, uric acid, and xanthine were purchased from Sigma (St. Louis, MO, U.S.A). KH_2PO_4 and Na_2CO_3, HCl

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(37%) were pro analysi grade, from Merck (Darmstadt, Germany). HPLC grade methanol and acetonitrile were obtained from Riedel-de-Haën (AG, Seelze, Germany). Bis de-ionised water was used throughout analysis.

HPLC Instrumentation

The chromatographic system operating in isocratic mode, consisted of the commercial components: a Shimadzu (Kyoto, Japan) LC-10A pump, an SSI 500 variable UV/VIS detector (SSI, State College, PA, U.S.A.), operating at 200 nm and a sensitivity setting of 0.002 (AUFS), a 7161 Rheodyne (California, U.S.A.) injection valve with a 20 μL loop. A Hewlett-Packard (Avondale, PA, U.S.A.) HP 3396 Series II integrator was used for quantitative determination of eluted peaks.

The analytical column, a Kromasil C_8, 250 mm × 4 mm ID, 5 μm, was purchased from MZ Analysentechnik (Mainz, Germany).

A glass vacuum-filtration apparatus obtained from Alltech Associates was employed for the filtration of mobile phase, using 0.2 μm membrane filters obtained from Schleicher and Schuell (Dassel, Germany).

Degassing of solvents was achieved by sonication in a Transonic 460/H Ultrasonic bath (Elma, Germany) prior to use. A Glass-col, Terre Haute 47802 small vortexer and a Hermle centrifuge, model Z 230 (B. Hermle, Gosheim, Germany) were employed for sample pre-treatment.

UV spectra for selecting the working wavelength of detection were taken using a Varian DMS 100S UV/VIS double-beam spectrophotometer.

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Chromatographic Conditions

The mobile phase of 10 mmol/L KH_2PO_4 was chosen in terms of peak shape, column efficiency, chromatographic analysis time, selectivity, and resolution. The analytical column was a Kromasil C_8, 250 mm × 4 mm ID, 5 μm. Inlet pressure observed with the eluent system, at a flow rate 0.8 mL/min, was 110–120 kg/cm^2. UV detection was performed at 200 nm.

Standard Solutions

Stock solutions of creatine and creatinine, at concentrations of 100.0 ng/μL, were prepared in water with 0.5 mL HCl (37%) in 100 mL and stored, refrigerated, at 4°C. These solutions were found to be stable for one month. Uric acid solution was prepared in 0.01% Na_2CO_3 to increase dissolution speed and solution stability. Xanthine stock 40 ng/μL solution was prepared in water and found to be stable throughout analyses. Fifteen working aqueous

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solutions were prepared from stocks at concentrations covering the range from 0.2 pg/µL to 15.0 ng/µL.

Sample Preparation

Serum samples were provided from a State Hospital (Blood Donation Unit). A 500 mL volume of pool serum sample was treated with 1 mL acetonitrile to precipitate proteins. After centrifugation at 3500 rpm for 15 min, acetonitrile was removed by evaporation. The residual was diluted ten to twenty times with deionized water and spiked, appropriately, with stock solutions to provide final concentrations of 0.09, 0,17, 0.33, 0.46, 1.0, and 1.67 ng/µL.

Human urine samples were collected from healthy volunteers and stored at −20°C until analysis. These were filtered from 0.2 µm membrane filters to remove cells and other particulate matter. Clarified urine samples were diluted 400 to 500 times with deionized water. Spiked samples were prepared by adding the appropriate volume of stock solutions to yield the final concentrations of 0.09, 0,17, 0.33, 0.46, 1.0, and 1.67 ng/µL.

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RESULTS AND DISCUSSION

Analytical Variables

Optimised chromatographic conditions were set and the statistical evaluation of the proposed method was performed according to the following parameters:

- Calibration data and analysis time.
- Working range and sensitivity.
- Precision and accuracy.
- Application to biological fluids: blood serum and urine.

Calibration Data and Analysis Time

The following retention times are achieved under the chromatographic conditions developed in the present assay: 2.631 min for Creatine, 3.400 min for Creatinine, 4.645 min for Uric Acid, and 7.014 min. for Xanthine, as shown in the chromatogram illustrated in Figure 2. Resolution factors for examined compounds are in the range 1.4 to 3.9.

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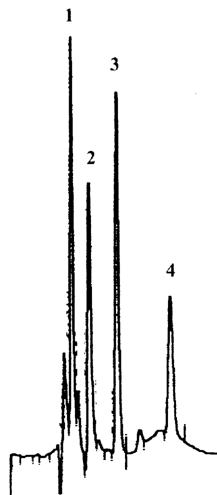

Figure 2. High performance liquid chromatogram of standard solution containing: (1) creatine 2.631 min, (2) creatinine 3400 min, (3) uric acid 4.645 min, and (4) xanthine 7.014 min. Chromatographic conditions are described in the text.

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Calibration of the method was performed by injection of standards, covering the entire working range. The sensitivity setting of the UV-VIS detector was adjusted at 0.002 AUFS. Each sample was injected six times.

Linearity of the standards was demonstrated by measuring different concentratons in the range from 0.2 pg/μL to 15.0 ng/μL. Linear relationships between absolute injected amount or concentration of the examined compounds and peak areas were observed. The results of the statistical treatment of calibration data are summarised in Table 1.

Working Range and Sensitivity

The minimum detectable concentration LOD was defined as a peak height that produces three times of baseline noise at 0.0005 AUFS. The LOQ was the lowest concentration of calibration standards with acceptable precision and accuracy.

The limits of detection were 0.2 mg/L for creatine and creatinine, 1 μg/L for uric acid, and 0.3 μg/L for xanthine, while the limits of quantitation were 0.5 μg/L for creatine and creatinine, 3 μg/L for uric acid, and 1 μg/L for xanthine. A rectilinear relationship was observed up to 2 ng/μL for creatine and creatinine, 12 ng/μL for uric acid, and 5 ng/μL for xanthine.

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Table 1. Calibration Data for Simultaneous Determination of Creatine, Creatinine, Uric Acid, and Xanthine

Parameter	Units	Creatine	Creatinine	Uric Acid	Xanthine
Linearity range	ng/μL	$0.5 \times 10^{-3} - 2.0$	$0.5 \times 10^{-3} - 2.0$	$3 \times 10^{-3} - 12.0$	$1 \times 10^{-3} - 5.0$
Slope	AIU/ng	1942716 ± 19653.38	3050737 ± 36146.2	7499413 ± 333604.1	340764.4 ± 42705.97
Intercept		170582.9 ± 12736.85	715408.8 ± 23425.41	288432.9 ± 216200.1	32098.13 ± 27676.63
Correlation coefficient		0.99995	0.99993	0.9990	0.992
LOD	μg/L	0.2	0.2	1.0	0.3

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Method Validation: Accuracy, Precision, Stability

The precision of the method, based on within-day repeatability, was performed by replicate injections ($n = 8$) of three standard solutions covering different concentration levels: low, medium, and high, where peak areas were measured. Statistical evaluation revealed relative standard deviations at different values. The reproducibility (day-to-day variation) of the method was established using the same concentration range as above. A triplicate determination of each concentration was conducted during routine operation of the system over a period of eight consecutive days. Reproducibility and repeatability results are illustrated in Table 2.

Accuracy was determined by replicate analysis of three different concentration levels (0.1, 0.5, and 1.0 ng/μL) and calculating the recoveries of actually found versus spiked values.

The stability of creatine, creatinine, uric acid, and xanthine solutions was verified by storing standard solutions, refrigerated, for a period of five weeks. Concentrations were measured periodically (one, two, three, four, and five

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Table 2. Day-to-Day (Over a Period of 8 Consecutive Days) and Within-Day ($n = 8$) Precision and Accuracy Study for Determination of Creatine, Creatinine, Uric Acid, and Xanthine

	Within-Day			Day-to-Day		
Added (ng)	Found ± SD (ng)	RSD	Recovery %	Found ± SD (ng)	RSD	Recovery %
Creatine						
2	1.92 ± 0.08	4.2	96.0	1.90 ± 0.14	7.4	95.0
10	9.76 ± 0.19	1.9	97.6	9.68 ± 0.43	4.4	96.8
20	20.60 ± 1.05	5.1	103.0	20.4 ± 1.0	4.9	102.0
Creatinine						
2	2.05 ± 0.08	3.9	102.5	2.10 ± 0.09	4.3	105.0
10	9.73 ± 0.40	4.1	97.3	9.80 ± 0.42	4.3	98.0
20	19.60 ± 0.64	3.3	98.0	19.44 ± 1.4	7.2	97.2
Uric Acid						
2	1.97 ± 0.06	3.1	98.5	1.96 ± 0.12	6.1	98.0
10	9.96 ± 0.04	0.4	99.6	9.65 ± 0.34	3.5	96.5
20	19.88 ± 0.80	4.0	99.4	19.50 ± 0.84	4.3	97.5
Xanthine						
2	1.95 ± 0.07	3.6	97.5	1.94 ± 0.11	5.7	97.0
10	10.43 ± 0.14	1.3	104.3	10.42 ± 0.61	5.8	104.2
20	19.71 ± 0.87	4.3	98.6	20.40 ± 1.00	4.9	102.0

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weeks). Dilute solutions were stable for two weeks, while stocks were stable for a period of one month.

Serum and urine samples containing different concentrations of creatine, creatinine, uric acid, and xanthine were found to be stable for one month without significant decomposition of the analytes when stored deep frozen.

Application to Biofluids

Serum

Pooled human blood serum was diluted 10 to 20 times after proteins precipitation with CH_3CN and spiked to give final concentration levels of 0.09, 0,17, 0.33, 0.46, 1.0, and 1.67 ng/mL.

The calibration curves were constructed from linear regression analysis of the peak area ratios versus the nominal concentrations of the calibration standards. Correlation coefficients were in the range 0.990–0.998. Calibration data are tabulated in Table 3. A high performance liquid chromatogram of creatine, creatinine, uric acid, and xanthine in human blood serum, is shown in Figure 3. No interference from endogenous compounds from sample matrix was noticed.

The concentrations of uremic toxins in pooled serum sample, as determined by application of a standard addition technique, were found to be 0.53 mg/dL for creatine, 0.17 mg/dL for creatinine, 0.66 mg/dL for uric acid, and 0.04 mg/dL for xanthine.

Urine

Pooled urine samples were simply diluted 400 to 500 times. In this way, any interference from endogenous compounds from sample matrix was eliminated allowing the determination of analytes, as shown in the chromatogram illustrated in Figure 4. The standard addition technique was applied by adding analytes at concentration levels of 0.09, 0,17, 0.33, 0.46, 1.0, and 1.67 ng/μL.

The calibration curves were constructed from linear regression analysis of the peak area ratios versus the nominal concentrations of the calibration standards. Correlation coefficients ranged from 0.986 to 0.9995. Calibration data are presented in Table 3.

The concentrations of uremic toxins in pooled urine sample were calculated by extrapolating the calibration curve, and found to be 63.7 mg/dL for creatine, 83.5 mg/dL for creatinine, 26.2 mg/dL for uric acid, and 0.4 mg/dL for xanthine.

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Table 3. Calibration Data for Creatine, Creatinine, Uric Acid, and Xanthine Determination in Human Serum and Urine

Parameter	Units	Creatine	Creatinine	Uric Acid	Xanthine
Serum					
Slope	AIU/ng	7342409 ± 904040.1	4128269 ± 125850.5	5682404 ± 237831.5	12152554 ± 1000505
Intercept		3886617 ± 299199.5	349349.6 ± 104620.1	1865215 ± 216369.2	274067.5 ± 910217.8
Correlation coefficient		0.992	0.998	0.997	0.990
Urine					
Slope	AIU/ng	2880940 ± 90112.01	1824589 ± 221569.1	2301321 ± 64874.28	6777916 ± 320140.2
Intercept		4585935 ± 91740.34	3047007 ± 196257.6	1207319 ± 37680.18	68804.58 ± 317193.4
Correlation coefficient		0.9995	0.986	0.9992	0.998

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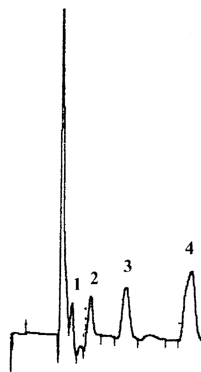

Figure 3. High performance liquid chromatogram of spiked human blood serum sample (after 20 fold dilution) containing: (1) Creatine 2.760 min, (2) Creatinine 3.615 min, (3) Uric acid 5.232 min, and (4) Xanthine 7.488 min. Chromatographic conditions are described in the text.

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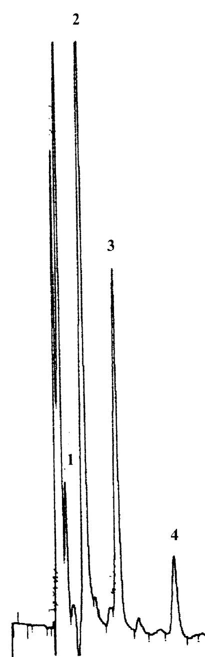

Figure 4. High performance liquid chromatogram of urine sample (after 400 fold dilution) containing: (1) Creatine 2.774 min, (2) Creatinine 3.675 min, (3) Uric acid 5.365 min, and (4) Xanthine 7.494 min. Chromatographic conditions are described in the text.

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The precision and accuracy studies of biological samples were conducted by spiking blood serum and urine samples with three known concentrations of the compounds, and then, by comparing obtained results with those calculated from regression equations. Results of recovery studies for serum and for urine samples are given in Table 4. Each value represents the mean of six measurements carried out.

Comparison with Conventional Routine Methods

Creatinine levels in nine urine samples from healthy volunteers were measured by Jaffé colorimetry in a State Hospital Biochemical Laboratory, and results were correlated with those obtained from the developed method. The respective uric acid levels were determined by the enzymatic procedure with the addition of uricase, and measurement of produced hydrogen peroxide by catalase or peroxidase. Correlation results are presented in Table 5. As proven by experimental t-test value, no statistically significant difference is observed between the results obtained by the two different methods.

Table 4. Recovery of Creatine, Creatinine, Uric Acid, and Xanthine from Human Blood Serum and Urine

	Serum			Urine		
Added (ng)	Found ± SD (ng)	RSD	R (%)	Found ± SD (ng)	RSD	R (%)
Creatine						
3.3	3.2 ± 0.2	6.2	97.0	3.25 ± 0.15	4.6	98.5
9.2	9.4 ± 0.6	6.4	102.2	9.6 ± 0.5	5.2	104.3
20.0	19.4 ± 0.8	4.1	97.0	19.95 ± 0.8	4.0	99.8
Creatinine						
3.3	3.25 ± 0.15	4.6	98.5	3.25 ± 0.2	6.2	98.5
9.2	9.2 ± 0.5	5.4	100.0	9.25 ± 0.35	3.8	100.5
20.0	20.5 ± 0.65	3.2	102.5	19.85 ± 0.5	2.5	99.2
Uric Acid						
3.3	3.2 ± 0.25	7.8	96.7	3.3 ± 0.15	4.5	100.0
9.2	9.6 ± 0.4	4.2	104.3	9.15 ± 0.2	2.2	99.5
20.0	20.1 ± 0.4	2.0	100.5	19.9 ± 0.3	1.5	99.5
Xanthine						
3.3	3.0 ± 0.2	6.7	90.9	3.4 ± 0.3	8.8	103.0
9.2	9.7 ± 0.7	7.2	105.4	9.7 ± 0.4	4.1	105.4
20.0	20.6 ± 0.75	3.6	103.0	19.7 ± 0.9	4.6	98.5

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Table 5. Correlation of Results Obtained by the Developed Method with Those by Conventional Routine Analytical Procedures for Creatinine and Uric Acid Determination in Urine

Creatinine (mg/dL)		Uric Acid (mg/dL)	
Colorimetric Method (Jaffé)	HPLC	Enzymatic Method	HPLC
110.0	107.1	20.0	22.5
181.0	182.1	47.0	44.4
52.0	52.2	14.0	10.6
173.0	173.1	44.0	36.0
146.0	148.9	4.2	5.5
95.5	87.4	10.0	11.2
81.5	83.4	62.5	58.0
54.0	40.8	19.0	20.0
107.5	107.6	37.5	35.6
$t = 1.122721$		$t = 1.403893$	
$t_{crit} = 2.306006$ ($P = 0.95$)		$t_{crit} = 2.306006$ ($P = 0.95$)	
$r = 0.9958$		$r = 0.9901$	

CONCLUSIONS

The accurate determination of creatinine and uric acid is very important in clinical diagnosis, as their levels are indicative of renal function. Their routine methods for their measurements, such as photometric methods or enzymatic methods, suffer from interference by various endogenous and exogenous compounds. The procedure described in the present paper is proposed as an alternative to these less specific methods. A simple, rapid, and direct method for the simultaneous determination of creatine, creatinine, uric acid, and xanthine has been developed. The statistical evaluation of the method performing day-to-day ($n = 8$) and within-day ($n = 8$) calibration yielded high accuracy and precision results. RSD values were in the range of 0.4 to 4.3% for within-day measurements and 3.5 to 7.4% for day-to-day precision measurements.

Sample manipulation was minimal, thus, the variations in the results are reduced. The limits of detection were 4 pg for creatine and creatinine, 20 pg for uric acid, and 6 pg for xanthine, while the limits of quantitation were 10 pg for creatine and creatinine, 60 pg for uric acid, and 20 pg for xanthine, when 20 μL were injected onto the column. The high sensitivity of this method enables the direct determination of creatinine and uric acid in diluted serum (10 to 20 fold dilution) and urine (400–500 fold dilution) samples. Percentage recoveries of analytes in spiked samples were in the range 91–105%.

Results for creatinine determination correlated well ($r = 0.9958$) with those obtained by Jaffé method, while results for uric acid determination correlated well

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(r = 0.9901) with those obtained by enzymatic method. Determination of creatine, creatinine, uric acid, and xanthine can be routinely performed in clinical assays using the developed method.

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