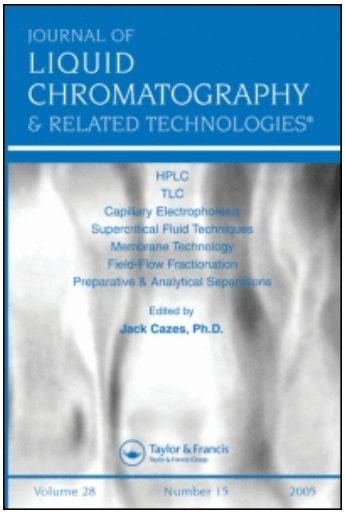

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ION-PAIR REVERSED-PHASE HPLC DETERMINATION OF CREATININE IN URINE

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ABSTRACT

A high performance liquid chromatography (HPLC) with isocratic ion-pair-reversed-phase separation and UV detection at 230 nm is proposed for the determination of creatinine in urine. The analysis is achieved on a C_{18}-Symmetry column (4 μm, 150mm x 3.9mm ID) using a mixture of 10 mM phosphate buffer pH=4, containing 3mM 1-octane-sulfonic acid, sodium salt and methanol (97:3). A flow-rate of 0.5mL/min and a column temperature of 30°C were employed. We validated the method for linearity, precision, and accuracy and the results were found to be excellent. The procedure proposed here is an alternative to less specific colorimetric methods.

INTRODUCTION

Urine creatinine is used to adjust the values of urinary biological indicators. Creatinine concentrations are very useful indexes for evaluating glomerular filtration rate and, in general, for indicating renal function. For the determination of creatinine , the unespecific Jaffé-method, although subject to perturbation by many interfering substances from endogenous and exogenous origin, is the most widely used.

Enzymatic assays have higher specificity but still suffer from interferences. To avoid these problems the isotope dilution-gas chromatography-mass spectrometry (ID-GC-MS)[1] and high-performance liquid chromatographic methods[2-7] include ion-exchange, reversed phase and ion-pair chromatography[8-9] were developed.

The purpose of this report is to describe an ion-pair HPLC method with UV detection that will permit the analysis of creatinine in human urine. This procedure is simple and rapid and it does not require any sample pretreatment other than dilution and filtration. The variations in the results are reduced because of the minimal sample manipulation and steps involved. Validation data for linearity, accuracy, precision and detection limit are presented.

EXPERIMENTAL

Chemical and Reagents

The creatinine used for preparation of the standard solutions (purity 99.7 ± 0.3 %) was Standard Reference Material (SRM914a) from the National Institute of Standards and Technology, Gaithersburg, MD 20899, U.S. Decane-, octane-, and hexanesulfonic acid (C10, C8, C6), sodium salts, were supplied by Sigma-Aldrich Química S.A. (Madrid, Spain). Methanol and Acetonitrile were of HPLC-grade obtained from Merck (Darmstadt, FRG). Other chemicals were of analytical grade. Deionized water was prepared with a Milli-Q apparatus (Millipore Bedford, MA, USA).

Standard Solutions

The creatinine stock-standard solution (1000 mg/L) was prepared monthly by dissolving creatinine (SRM 914a) in water and stored at 4°C. For HPLC measurements, two standards (10 mg/L and 20 mg/L) were prepared freshly each day with the chromatographic eluent as solvent.

The calibration range was 5 to 50 mg/L, obtained by dilution of stock-standard solution with HPLC eluent buffer. The calibration graph was based on peak areas and were linear over the concentration range investigated. Triplicate injections of each concentration were made.

Sample Preparation

Urine samples obtained from test subjects were centrifugated for 10 min at 2000 g and filtered through a Millex-HV 0.45 μm pore size filter (Millipore,

Bedford, MA,USA) and diluted 50-fold with mobile phase. A 20 µL aliquot was injected on the HPLC column. Urine samples were stable for several weeks when stored at -20ºC.

Instruments and Chromatographic Conditions

A Waters Model (Milford, MA,USA) 600-E instrument equipped with a Waters Model 484 UV detector was used. Quantification was based on integration of peak areas using a Waters Model 745B Integrator.

The separation was performed on a Symmetry C_{18} (5 µm, 150mm x 3.9mm ID) (Waters, Mildford, MA, USA). The mobile phase was phosphate buffer 10mM with 3 mM 1-octanesulfonic acid, sodium salt, pH = 4, and methanol (97:3). Before use, the mobile phase was always filtered through an HA 0.45 µm pore size filter (Millipore, Bedford, MA, USA) and degassed by ultrasonication.

The flow rate was 0.5 mL/min and the retention time for creatinine was 4.1 min. The column was equilibrated with the mobile phase for at least 30 min at the beginning of each day. The analysis was run at 30°C and the detection wavelength was set at 230 nm.

The purity of the creatinine peak was tested by comparison of the peak areas obtained at wavelengths of 218, 230, and 254 nm.

RESULTS

Optimization of Chromatographic Conditions

We tested different variables to optimize the analysis of the creatinine under isocratic conditions.

Figure 1 illustrates the relationship between the creatinine retention time and the alkyl chain length of the pairing-ion agent (C6, C8, C10) with a fixed concentration 3mM at different methanol percentages. The aqueous component of the mobile phase was buffered at pH 4 with a 10mM phosphate buffer. Increasing the percentage of methanol in the mobile phase allowed a more rapid elution of creatinine. Similar studies were carried out with acetonitrile as organic modifier. On the basis of these studies we decided to select methanol (3%) in this determination.

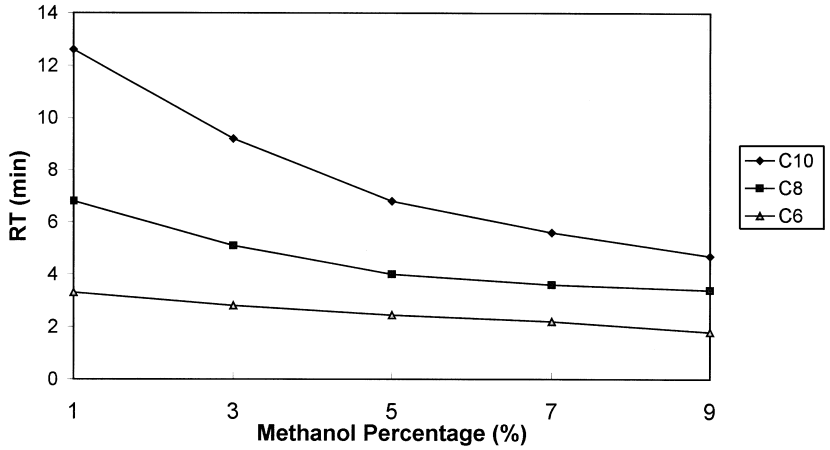

Figure 1. Relation between creatinine retention time (RT) and pairing-ion alkyl chain length (C6, C8 y C10) at different methanol percentages. The mobile phase was buffered at pH = 4 with a 10mM phosphate buffer containing 3 mM pairing-ion concentration. Column temperature was set at 25ºC.

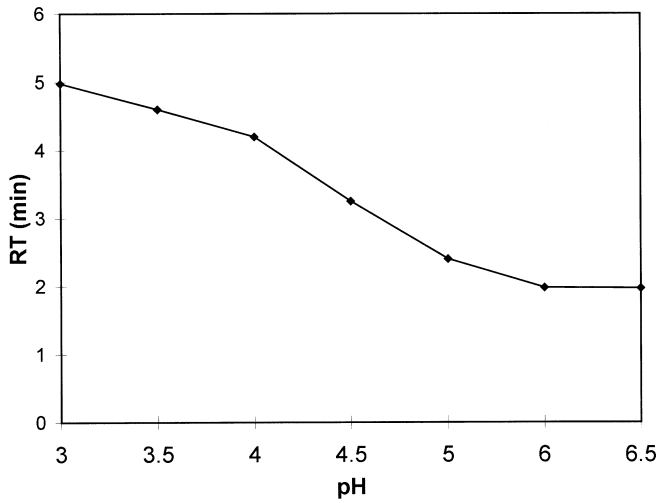

Figure 2. Influence of the aqueous solution pH on the creatinine retention time. 1-octane-sulfonic acid, sodium salt concentration was 3 mM with acetic/acetate eluent in the pH = 4.5, KH_2PO_4 eluent in the pH range 3-6.5 with 3% of methanol. Column temperature 30ºC.

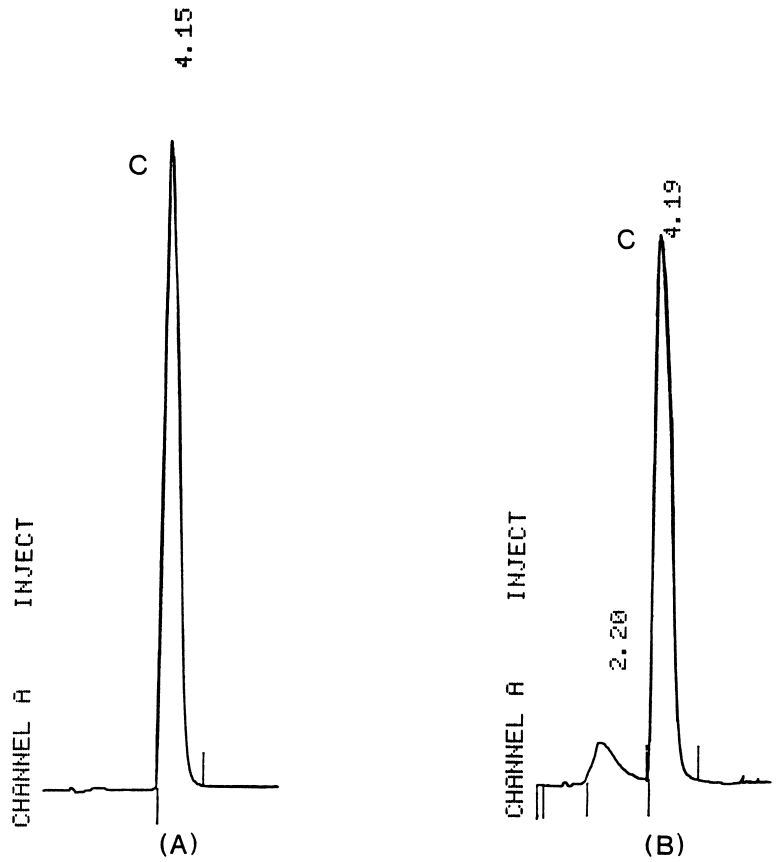

Figure 3. Chromatogram of creatinine using UV detection (λ= 230 nm), flow rate 0.5 mL/min, injection volumen 20 µL, column temperature 30ºC and a mobile phase of 10 mM phosphate buffer pH = 4 containing 3 mM 1-octane-sulfonic acid, sodium salt and methanol (97:3). A is the chromatogram of creatinine standard and B of a urine sample.

The retention time of creatinine decreased with the chain length of the pairing-ion molecules from C10 to C6. We observed a nonlinear relationship between retention time of creatinine and paired-ion concentration (3 to 10 mM) as described Tomlinson and al.[10]

Figure 2 shows the variation of creatinine retention time as the pH of the mobile phase ranged from 3.0 to 6.5. As expected, the retention time decreased with increasing pH. The retention time increased when the pH was lowered from 4.5 to 3.0, because the creatinine is a weak base (Pk_a 5.02) and remained in cationic form and produced a "paired-ion" with the counter-ion. In the 5.0-

6.5 pH range, the molecule is in neutral form and the retention time does not show appreciable variations. We decided to adopt the pH 4.0. In order to search for better chromatographic conditions, the effects of the buffer concentrations (10 to 50 mM) and column temperature (25-50°C) were investigated and we have verified that there is little influence of these parameters on the creatine retention time.

According to these studies, the best determination of creatinine in urine was obtained using the chromatographic conditions specified under Experimental.

The chromatograms resulting from the injection of pure standard and urine under the chromatographic conditions finally adopted are presented in Figure 3. Under these conditions there were no other endogenous urinary components that can interfere with the peak of creatinine.

Analytical Variables

Linearity

Linearity of the standards was demostrated by measuring different concentrations in the ranges 5-50 mg/L. Linear relationships between the creatinine peak areas and the concentrations tested were found. The equation calculated was: $y = 0.133\ x + 0.072$, with a correlation coefficient ($r>0.999$). The standard addition method was used to check for chemical interferences in the determination of creatinine. The equation calculated was: $y = 0.128\ x + 0.780$; ($r>0.998$).

The slopes found for the calibration and standard addition graphs were similar.

Statistical analysis by the t-test showed that the slope values are not statistically significant ($t= 0.033$, $p= 0.98$).

Analytical Recovery, Precision, and Accuracy

The analytical recovery was evaluated by assaying urine samples spiked with different amounts of creatinine standard ranging from 2.5 to 25 mg/L. Replicat analysis (n=5) at each concentration were made. The main recovery was 95.13 ± 2.76 (RSD= 2.90%).

The interday precision and accuracy were asseassed by analysing urine samples containing different concentrations of creatinine five times per day during one week. The results are given in Table 1.

Table 1

Inter-Day Precision and Accuracy

Conc. Added (mg/L)	Conc. Found (mg/L)*	RSD (%)	RE (%)
2.5	2.37 ± 0.47	19.93	4.86
5	4.42 ± 0.47	10.64	11.49
7.5	7.26 ± 0.06	0.90	3.18
10	9.39 ± 0.40	4.24	6.01
12.5	11.92 ± 0.39	3.21	4.66
15	14.42 ± 0.29	2.03	3.84
17.5	16.88 ± 0.39	2.30	3.53
20	19.31 ± 0.31	1.60	3.42
25	24.43 ± 0.09	0.33	2.24

* Mean ± SD; (n = 5).

The detection limit was 0.70 mg/L with a 20 μL injection and was determined from the calibration curves according to the method described by Miller and Miller.[11] The results indicate that this ion-pair reversed-phase HPLC method is suitable for the determination of creatinine in human urine.

CONCLUSION

The described ion-pair HPLC method has been shown to be linear, precise, accurate, and specific. Thus, this method is accurate and reliable for the determination of creatinine in urine samples.

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