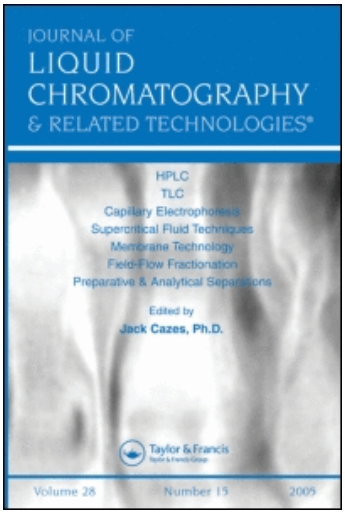

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Luz Ma. Del-Razo[a]; Pedro Jauge[a]
[a] Departmento de Farmacologia y Toxicologia, Mexico, D.F.

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QUANTITATION OF CREATININE IN URINE AND PLASMA SAMPLES BY REVERSED PHASE HPLC

Luz Ma. Del-Razo and Pedro Jauge*

Departmento de Farmacologia y Toxicologia
CINVESTAV-IPN Apartado Postal 14-740
07000 Mexico 14, D.F.

ABSTRACT

Creatinine determination in urine and plasma affords an index of the renal function. Reversed-phase high pressure liquid chromatography was used for the separation and quantitation of creatinine in normal and arsenic exposed human urine samples. Acetonitrile/water (1:1) was the mobile phase. The method was compared with the Jaffé alkaline picrate reaction. Results show that the HPLC procedure has high reproducibility and samples are stable at the storage conditions. Plasma samples required deproteinization and extraction with CH_3CN prior to HPLC analysis, while urine samples required only centrifugation.

INTRODUCTION

The formation of creatinine (CT) is required to allow the excretion of creatine. Free creatinine is present both in blood and in urine. The urinary excretion of CT in 24 hs is constant from day to day in a given subject. The ratio between excreted CT in this

*To whom correspondence should be addressed.

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period and the body weight is almost constant for different individuals with the same age and sex. Also, the clinical determination of CT in plasma and urine affords a quantitative index of the renal function[1].

Hojo[2,3] suggests for selenium, that the expression ng Se/mg of CT in single-void urine samples is not only a better expression of selenium level, but also a more effective indicator of selenium status in humans, in comparison with ng Se/ml of urine.

In a human population, chronically exposed to arsenic through drinking water, we have obtained the first void urine sample, due to difficulties in 24 hs collection[4]. In this case, it was convenient to know CT levels in urine, to avoid doubts related with urine sample collection.

Clinical procedures for the determination of creatinine are based on the Jaffé method, in which CT reacts with alkaline picrate to yield an ambar-yellow chromogen. This method, although rapid, does not posses the desired specificity and accuracy. To increase the specificity, it is recommended differential absorbance measurements using an interval of 5 min., due to different reaction rates between CT and others chromogens present in the sample with the alkaline picrate[5]. The preparation and stability of reagents of a kinetic enzymatic method[6] reduces the potential for routine analysis.

Actually, the use of high pressure liquid chromatography (HPLC) becomes more important. Cationic exchange[7,8], normal phase[9] and reverse-phase[10-14] HPLC are used for serum, plasma and urine.

In this study, the aplication of reversed-phase HPLC for the determination of creatinine in urine and plasma samples using CH_3CN/H_2O (1:1) as mobile phase is considered, paying attention to sample treatments. We have also compared the HPLC procedure with the Jaffé alkaline picrate method.

MATERIALS and METHODS

Reagents and Solvents.

All reagents were Analytical Reagent Grade. Creatinine -anhydrous- used as standard, was purchased from Sigma Chem. (St. Louis, USA) and used without further purification.

Acetonitrile, LiChrosolv^{R}, HPLC grade, was purchased from Merck (Darmstadt, G.F.R.). Water was purified with a Milli-RO 15 Reagent Grade water system (Millipore Corp., USA) and glass-redestilled.

Apparatus.

A Varian High Pressure Liquid Chromatograph model 8500, coupled with a VariChrom variable wavelenght Spectrophotometer (8 µl cell) and a Varian model 9176 Recorder were used for chromatographic determinations. Samples were introduced with a 10 µl syringe (Hamilton), through a Varian AeroGrapgh High Pressure Septumless Liquid Chromatography Injector.

pH determinations were carried out with a SelectIon 2000 Ion Analyzer (Beckman Instr., Inc., USA) using a Beckman Laboratory Combination pH Electrode. Samples were centrifuged with a Beckman model TJ-6 Centrifuge, coupled with a refrigeration unit.

Colorimetric determinations were carried out with a Coleman 44 Spectrophotometer (Perkin Elmer, Illinois, USA).

Chromatographic Conditions.

A prepacked MicroPack MCH-10 stainless steel column (250 x 4 mm i.d., 6647 theoretical plates) and a column guard packed with Vydac RP (40 x 4 mm) supplied by Varian were used.

Solvents - filtered and degassed by vacuum - were used at an elution rate of 30 ml/h. UV detector was set at 210 nm. An slit of 16 and a sensitivity of 0.1 AUFS was used. Chart speed was 2.5 cm/min.

Urine and Plasma Samples.

Normal urine and plasma specimens were obtained from laboratory personnel. First void urine samples were used. Blood samples

were obtained by venous puncture. Urine and Plasma samples were also used from individuals chronically exposed to arsenic[4]. Samples were stored at -10°C if not analyzed inmediatly.

RESULTS and DISCUSSION

Fig-1- shows typical HPLC chromatograms of urine and plasma samples obtained using the described procedure. In order to improve the creatinine separation from other components in samples, K_2HPO_4 0.1 M buffers at differents pH (2.3 - 8.2) and a mixture of acetonitrile/water (1:1) as mobile phases were used, with a reversed-phase C_{18} column.

In the table -1- is shown retention times and relative peak area for differents mobile phases used.

The results show that CH_3CN/H_2O (1:1) is an adequate mobile phase. Retention time was 67 ± 5 s. A relative peak area in relation to K_2HPO_4 0.1 M buffer (pH = 2.3) was 88.9%. This mobile phase has also the advantage to extend the column life.

TABLE -1-

Mobile Phase K_2HPO_4 0.1 M Buffer pH	Retention Time seg.	Relative Peak Area %
2.3	64 ± 3	100.0
4.4	76 ± 5	92.6
6.1	103 ± 7	17.2
8.2	121 ± 6	6.1
CH_3CN/H_2O (1:1)	67 ± 5	88.9

Chromatographic conditions as above described

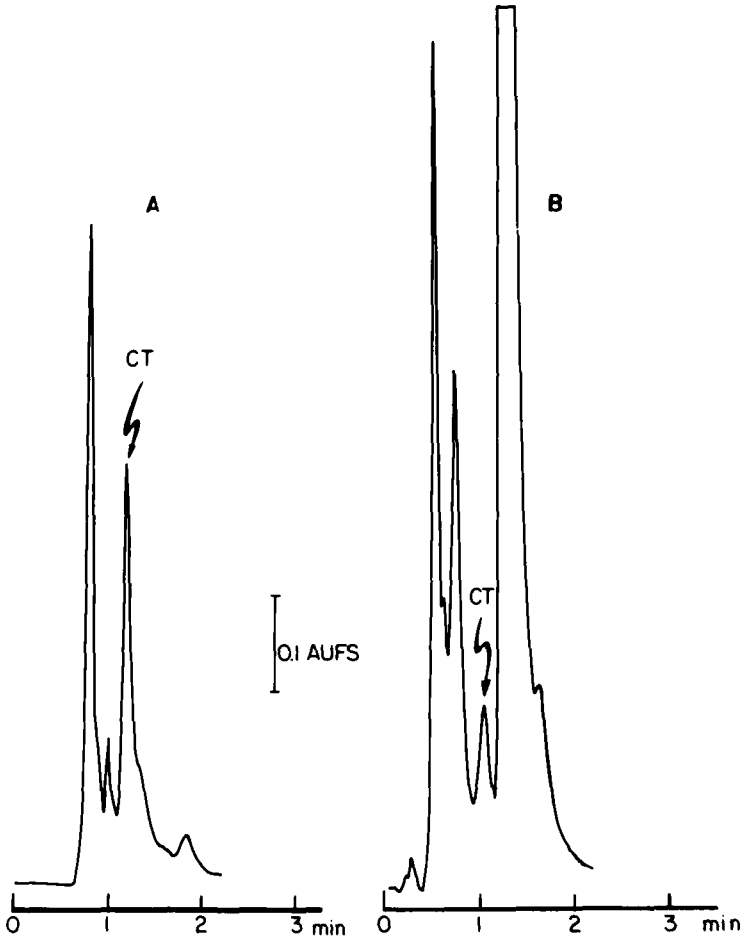

FIGURE -1- Reversed-Phase HPLC Chromatograms

Mobile phase: CH_3CN/H_2O (1:1) 30 ml/h
Detector: UV 210 nm.

A) Urine sample (0.1 µl)
B) Plasma sample (3 µl) (CH_3CN extract)

Good linearity was observed for peak area at the chromatographic conditions. Fig-2- (m = 5.67; r = 0.993). The identification of the creatinine peak in biological samples was done by the stopped-flow UV scanning technique[15], besides the retention time.

Urine Samples.

Urine samples were centrifuged and filtered through Millipore (0.45 μm) filter to extend the life of the column. The filtrate was injected directly to the column. The standard addition method was used to check for chemical interferences in the quantitation of creatinine. The slope found using this method (m = 5.85; r = 0.997) is similar to the calibration curve. Fig-3-.

Plasma Samples.

Prior to injection to the liquid chromatograph, plasma samples were deproteinized and creatinine was extracted with acetonitrile, according to Chiou et al.[8].

We have found that the recovery of creatinine from plasma samples using this procedure is 78.2 ± 1.9%. This was confirmed with the standard addition method.

Stability of Samples.

In order to know if the storage conditions have influence in the determination of creatinine, we have carried out a similar scheme previously described[16].

We have not found significant differences after 12 weeks in urine and plasma samples (% var. = 4.7 ± 1.8).

HPLC vs Jaffé Method.

HPLC procedure was compared with the Jaffé alkaline picrate reaction in urine samples from a population chronically exposed to arsenic through drinking water[4].

Results are shown in the Table -2-.

The creatinine concentration estimated by the HPLC procedure is lower than the value found when the Jaffé method is used by a factor of 0.65 ± 0.09. This is in agreement with the results of Chiou et al[8].

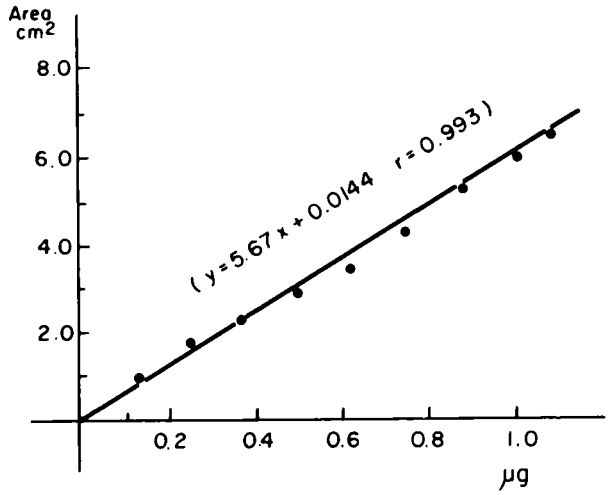

FIGURE -2- Creatinine Calibration Curve

Column: MicroPack MCH-10
Mobile phase: CH_3CN/H_2O (1:1)
Elution rate: 30 ml/h
Detector: UV 210 nm

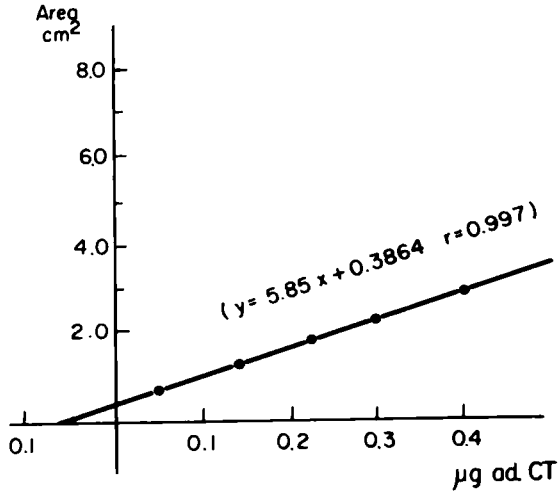

FIGURE -3- Standard Addition Method for Creatinine in Urine.

TABLE 2

Urine Sample	HPLC Method	Jaffé Method	HPLC/Jaffé ratio
	CT mg/ml		
A-18	1.15	1.65	0.70
A-31	0.50	0.82	0.61
A-32	0.61	1.28	0.46
A-42	0.92	1.25	0.74
A-52	0.90	1.46	0.62
B-17	0.70	1.08	0.65
B-26	0.60	0.74	0.80
C-24	1.01	1.50	0.67
C-31	0.45	0.84	0.54
C-47	1.26	1.90	0.66
			$\bar{X} = 0.65 \pm 0.09$

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