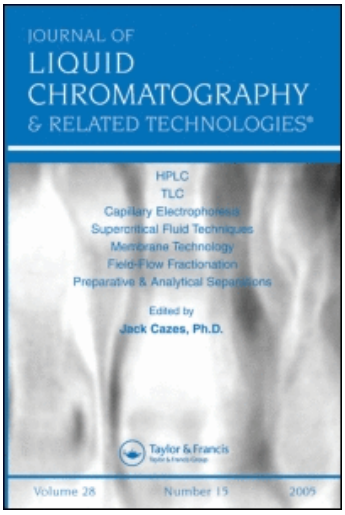

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HPLC Method for the Assay of Creatinine in Urine

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HPLC METHOD FOR THE ASSAY OF CREATININE IN URINE

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ABSTRACT

A simple and rapid HPLC method has been developed for the determination of creatinine in urine.
Urine was diluted (1:50 v/v) with water and injected directly.The chromatographic separation was accomplished with butylamine-phosphoric acid buffer at a flow rate of 1.2 ml/min.The HPLC column was a cartridge packed with 3 μm Econosphere C18. The UV detection wavelength was 230 nm. The intra and inter assay coefficient of variation were less than 5.2 %.The method was validated by comparing data on 232 urines with that obtained by the Jaffe method.It is suitable for routine determination of creatinine in urine as a measure of excretion rate, and is presently being used to improve the quality of the data from drug testing programs.

INTRODUCTION

Creatinine is the end product of creatine metabolism. Measurement of creatinine levels in urine and other biological fluids is extensively used as an indicator of renal function. It can also be used to estimate excretion rate[1,5]. Currently the method most widely employed for the creatinine determination in clinical laboratories is the Jaffe alkaline picrate procedure[2]. The Jaffe method requires several steps and it was reported that it can give overestimated values of creatinine due to interferences by endogenous and exogenous "pseudo-creatinine" chromogens[1].

HPLC assay of creatinine was proposed as a good substitute. Approaches included cation exchange columns[3], ion-pairing reagents[4,5], normal-phase chromatography with basic additive[6], reverse-phase chromatography with phosphate buffers[8,9] and reverse-phase chromatography with ammonium acetate buffers[7,10]. Urine was usually diluted with distilled water and injected. Only in one procedure[9] acetonitrile was added to the diluted urine and the solution was then centrifuged. Creatinine was usually detected at 235 nm (absorbance maximum) and in one case[9] at 200 nm.

An external standard method[3,4,5,8,10] and an internal standard method[6,7,9] were reported with precision and recovery of 1.3-4.9 % and 95.3-104.0 % respectively.

Our objective was to develop a fast and reliable method suitable for the high sample load of a drug testing laboratory. The time of the analysis of the above mentioned methods was too long

(minimum > 5 min), and internal standard method added one step to sample preparation phase. As an obvious solution short cartridges with small particles packing and external standard method came to our attention.

MATERIALS AND METHODS

Reagents and Chemicals

All reagents were analytical grade and were used without further purification.Water was deionized by Nanopure deionizer (Millipore Corp., Bedford, MA, USA) in our laboratory and filtered (0.45 μm filter, Millipore Corp., Bedford, MA, USA).

Butylamine (99%+) and phosphoric acid (85%) were from Aldrich Chem. Co., Milwaukee, Wis, USA.

Creatinine standard solutions were from Sigma Diagnostics, St. Louis, Mo, USA. Buffer solution was prepared by adding to a 1000 ml of water 0.075 ml of 8.5 % phosphoric acid and 0.200 ml of 10 % butylamine solutions. The buffer was filtered through 0.45 μm Millipore filter.

Urine was diluted 1:50 v/v with deionized water.

(20 μl in 980 μl of water).

Chromatographic procedures

The HPLC 1090 with filter photometric detector and automatic detector was obtained from Hewlett-Packard Boeblingen FR Germany. The chromatographic signal was recorded by a Hewlett-Packard integrator model 3393A.

The analytical cartridge (30, 4.6 mm I.D.) was filled with Econosphere C18 (3 μm) from Alltech Ass.,Deerfield, IL, USA.

The guard column (10, 2 mm I.D.) was packed with Pellicular ODS (37-53 μm) packing from Whatman Inc. Clifton N.J., U.S.A.

The mobile phase was n-butylamine-phosphoric acid buffer. The flow rate was 1.2 ml/min.

Detection was by filter photometric detector at 230 nm. Separations were done at room temperature.

Creatinine from Sigma Diagnostic 3 mg/dl was used as external standard. The same standard was used in Jaffe method of creatinine analysis.The injection volume was 2 μl.

Peak purity analysis was performed on HPLC 1090 with diode array detector DAD 1040 and ChemStation from Hewlett-Packard Boeblingen, FR Germany.

Jaffe method

A commercial kit supplied by Sigma Diagnostics was used according to manufacturers directions.

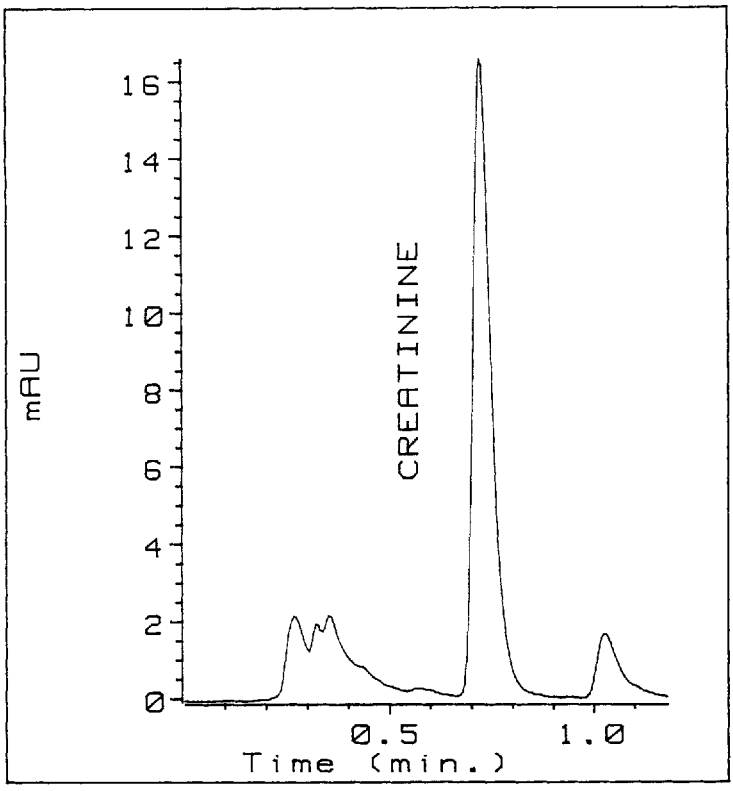

Figure 1. HPLC of diluted urine sample. Peak at 0.782 min is creatinine. Other peaks were not identified.

RESULTS AND DISCUSSION

A typical chromatogram is shown in figure 1. The creatinine peak is well resolved from the other peaks and it's retention time is quite reasonable for the analysis which has to be performed 1000 times per month.

The peak with $t_r = 0.725$ is creatinine. The identity of other peaks in the chromatogram were not of our interest.

Calibration curve was linear for creatinine ($y= 0.0235x+0.310$, $r^2=0.999$)over the concentration range of 50-500 mg/dl.

The quantification limit was determined by sequentially diluting the standard and sample with water and recording the signal at low attenuation (signal height of ten times the noise level). The limit was about 0.5 mg/dl with an injection volume of 1 µl and 1 : 50 dilution.

The peak purity of the creatinine peak was checked with a diode array detector. Forty samples were checked by three methods: comparison of the spectra at the beginning, apex and tail of the peak; comparison of spectra to the spectra of creatinine standard; and by the wavelength ratio method (3D Graph).The creatinine peak was pure in all of the samples.

Five samples and a standard solution were analyzed every 40 minutes up to the 360 minutes to check the stability of the sample and standard solutions. Analysis of variance showed that there was no significant difference in creatinine concentration for all these samples and standard solution even after six hours.

Interday and intraday variability was checked on 27 samples . The interday variability was equal or lower than the intraday variability.

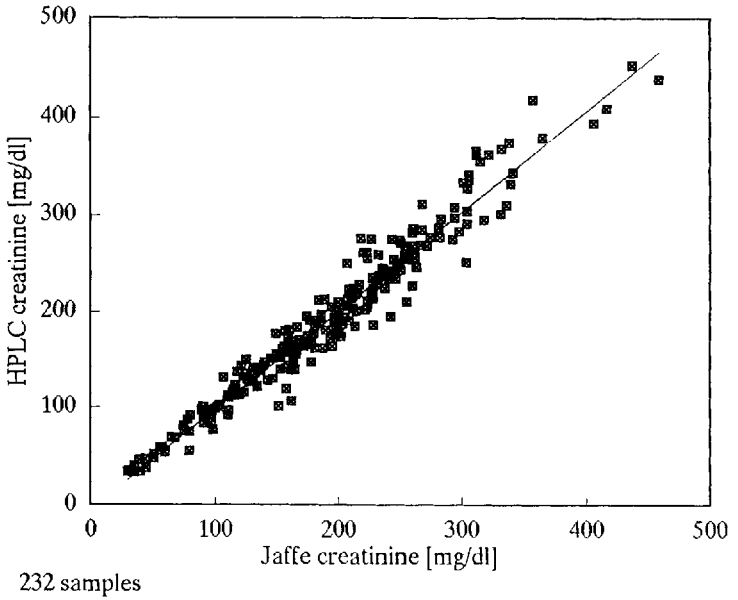

Figure 2. Comparison of creatinine measurements of 232 urine samples by HPLC and Jaffe methods.

To compare the HPLC method with the Jaffe method 232 urine samples from athletes were measured by both methods. Results are usually compared by using product-moment correlation (r) as a measure. In figure 2 usual plot of results of one method (Jaffe) versus results of another (HPLC) is shown.Correlation is very good (r=0.976).

That this measure is not the right one to assess if two methods are interchangeable has been demonstrated and interclass correlation (r_I) was suggested as a better measure of method

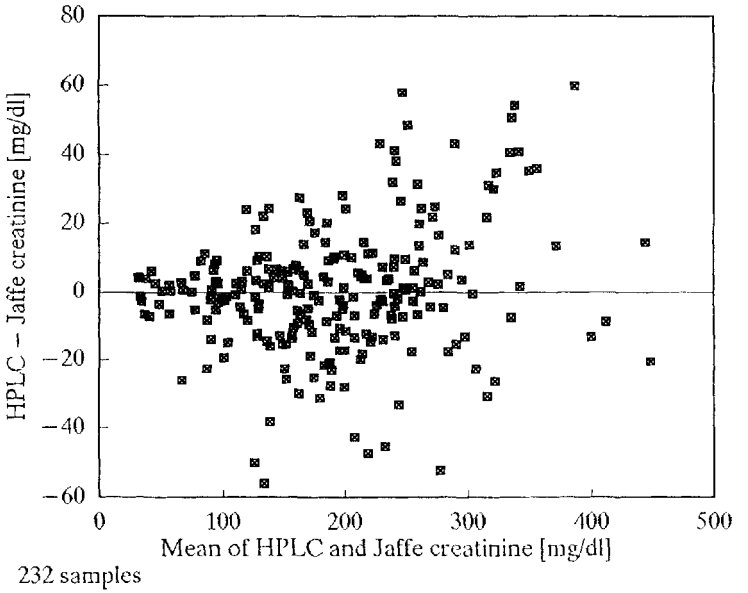

Figure 3. Relationship between difference [HPLC-Jaffe] and mean [(HPLC+Jaffe)/2)] assays of creatinine in 232 urine samples obtained by HPLC and Jaffe methods

interchangeability[12]. To use this measure one should first test for the presence of systematic bias between the two methods. In figure 3 the difference of readings between the two methods against the mean of the two methods is plotted.

It could be seen that there is no systematic bias and that we can use interclass correlation (r_I) to asses the agreement between the two methods. Meaningful agreement of two methods is attained if the lower limit of the 95% confidence interval is 0.75.

The results of our comparison are r_I of 0.975 and the lower limit of 95% of confidence interval for r_I was 0.969. The methods are therefore interchangeable.

In summary it could be concluded that the proposed method is a good substitute for Jaffe assay for creatinine. It is very fast and reliable, sample preparation is a simple one and the chromatographic assay is very fast (less then a 1 min), so an assay of 200 samples per day could be easily accomplished. Advantage over the other methods are short time of analysis due to the short column and high flow rate. Level of accuracy and precision is comparable to other methods. The method is presently used for correcting data for drug testing programs at the UCLA Olympic Laboratory.

We would like to express our gratitude to Dr Mario Panaque of UCLA School of Public Health for using DAD equipped HPLC in his laboratory.

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