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Note

High-performance liquid chromatographic assay of creatinine in human urine

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For several years we have been interested in the urinary concentrations of neurotransmitters and their metabolites in mental disease as a means of improving diagnosis and treatment. The most common way of reporting the 24-hour urinary concentration of neurotransmitters and their metabolites has been to express the value per milligram of creatinine. Thus, in order to obtain accurate ratios, a selective and accurate creatinine assay is imperative. The classical Jaffé alkaline picrate method [1], although rapid, has been reported [2] to be influenced by the presence of various organic compounds in the urine. Thus, the alkaline picrate creatinine determination does not possess the selectivity and the accuracy that we desired. By combining cation-exchange chromatography for the separation of creatinine from interfering, picrate-positive, compounds in urine and quantitation of the isolated creatinine by the alkaline picrate method, the accuracy of the determination can be improved [2]. This procedure has been automated [3] and represents a highly precise and quick means for the determination of urinary creatinine. However, the accuracy of this procedure has been reported [2—4] to be only slightly better than the classical Jaffé method. The pH control, the temperature control, and the precise timing of the alkaline picrate procedure [2] reduces the accuracy of the method. Some alternative means for the quantitation of creatinine after it is removed from the cation-exchange column was necessary.

The ability of a reversed-phase high-performance liquid chromatographic (HPLC) column to separate polar molecules in an aqueous medium appeared ideally suited for the analysis of creatinine. Creatinine, because of its inherent ultraviolet absorbance, could easily be detected at 254 nm [5]. We modified the HPLC creatinine procedure of Lim et al. [5] through the addition of an internal standard, 4-aminomethylpyridine (4-AMP). We report our results in this communication.

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EXPERIMENTAL

Materials

A Beckman DB ultraviolet—visible spectrometer at 515 nm was employed for the alkaline picrate creatinine analysis. An LC-50 high-performance liquid chromatograph (Bioanalytical Systems) with a μBondapak C_{18} (10 μm, Waters Assoc., Milford, Mass., U.S.A.) reversed-phase analytical column (30 cm × 3.9 mm I.D.) was used for the separation of creatinine and the internal standard. Detection was facilitated by a fixed wavelength (254 nm) ultraviolet detector (Altex, Model 153 analytical detector). A Speed Vac concentrator (Savant) was connected to an automatic freeze-dryer (Virtis, Model 10-010) for the concentration of aqueous samples.

4-Aminomethylpyridine (4-AMP), 99% pure, was obtained from Aldrich (Milwaukee, Wisc., U.S.A.) and was used without further purification. Creatinine zinc chloride was obtained from Eastman Chemicals (Rochester, N.Y., U.S.A.) and was used without further purification.

Solutions

Water was glass-distilled from an alkaline permanganate solution and was passed through a 0.22 μm Millipore filter before being used.

Standard creatinine solution. Creatinine zinc chloride was dissolved in 0.1 *N* HCl to afford a 1.41 mg/ml solution.

Internal standard solution. 4-Aminomethylpyridine was dissolved in water to afford a 0.98 mg/ml solution.

Standard urine. A 24-hour urine pool from a normal, healthy male was used in all analyses.

Citric acid buffer (pH 3.0). To 40 mmole of citric acid and 20 mmole of Na_2HPO_4 was added 1.0 l of water. The pH of this solution is 3.0 without adjustment.

Mobile phase. A 0.5 *M* sodium acetate solution (pH 4.7) containing 10% (v/v) methanol (glass-distilled) was prepared and was filtered through a 0.22-μm filter prior to use.

Analytical procedure

Preparation of creatinine standards. Solution 1: 0.50 ml of standard urine. Solution 2: 0.50 ml of standard urine + 0.20 ml of standard creatinine solution. Solution 3: 0.50 ml of standard urine + 0.40 ml of standard creatinine solution. Solution 4: 0.50 ml of standard urine + 0.60 ml of standard creatinine solution.

Sample preparation [5]. To each creatinine standard solution and to each 0.50-ml urine sample was added 0.50 ml of the internal standard solution. To each solution were added 24.0 ml of water and 5.0 ml of citric acid buffer (pH 3.0). Duplicate 3.00 ml aliquots were taken from each solution for creatinine analysis.

Cation-exchange chromatography. Fresh AG 50W-X12 (Bio-Rad Labs., Richmond, Calif., U.S.A.) cation-exchange resin, 100 mg per sample, was added to 2.50 ml of a 2.5 *M* NaOH solution and was allowed to sit overnight at ambient temperature. Resin was added to a Pasteur pipette (5 in.) that was lightly plugged with glass wool. The sodium hydroxide solution was allowed

to drain completely through the resin. Each column was washed with 2.0 ml of water. Each column was activated by the passage of 4.0 ml of citric acid buffer (pH 3.0) through the column. To each column were added 3.0 ml of the appropriate sample solution. Each column was allowed to drain, and was washed with 4.0 ml of water. Creatinine and 4-AMP were eluted from the resin by the addition of 4.0 ml of 1.0 *M* sodium acetate to the column. The sodium acetate eluate was collected in a small test-tube and was concentrated under vacuum to approximately 0.5 ml in a Speed Vac concentrator. To each sample was added 1.0 ml of water.

HPLC analysis

A 20-μl loop injector valve was used to place each sample onto a μBondapak C_{18} (10 μm) analytical column. The flow-rate of the mobile phase through the column was 1.0 ml/min. Detection was effected by a fixed wavelength (254 nm) ultraviolet detector. Creatinine eluted from the column at 4.5 min while 4-AMP eluted at 5.5 min (see Fig. 1).

Quantitation

Using the creatinine/4-AMP peak height ratio from the standard solutions and plotting this ratio against the creatinine concentration (mg/ml) added

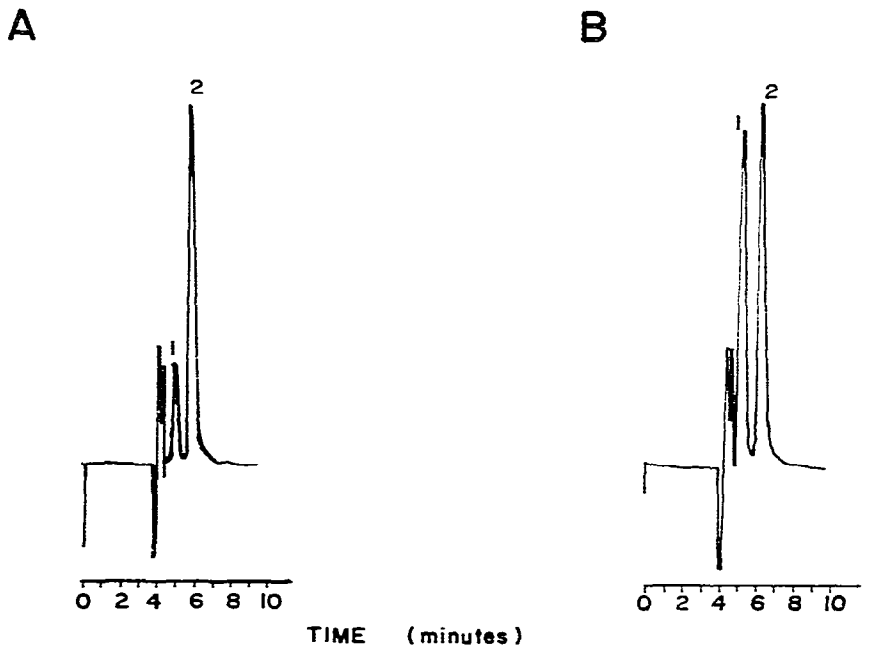

Fig. 1. Chromatograms of urinary creatinine. After addition of internal standard and passage through an ion-exchange column, the chromatograms obtained are shown for two different urinary concentrations of creatinine: (A) 0.83 mg/ml urine; (B) 2.57 mg/ml urine. 1 = Creatinine, 2 = internal standard, 4-aminomethylpyridine (4-AMP). Conditions: column, μBondapak C_{18} (10 μm); solvent, 0.5 *M* sodium acetate (pH 4.7) plus 10% methanol; flow-rate, 1.0 ml/min; detection at 254 nm.

to the standard urine, a least-squares line, $y = 0.35x + 0.33$, was fitted through the data points, correlation coefficient 0.993. The slope of the least-squares line was used for calculation of the creatinine concentration in the unknown urine samples.

Because of the relatively high urinary creatinine concentration in the 0.50—4.00 mg/ml range, the limit of detection by this method was not determined. However, this procedure afforded linearity over the range 0.02—6.15 mg/ml.

RESULTS AND DISCUSSION

The procedure of Lim et al [5] involved the separation of creatinine from urine by cation-exchange chromatography, elution of the creatinine from the column and HPLC reserved-phase analysis of the eluted creatinine with ultraviolet detection at 254 nm. Lim et al. [5] did not use an internal standard in this procedure.

We found that 4-AMP fits all of the qualifications of an acceptable internal standard [6]. Using a mobile phase of 0.5 *M* sodium acetate (pH 4.7) containing 10% (v/v) methanol at a flow-rate of 1.0 ml/min, creatinine had a retention time of 4.5 min while 4-AMP had a retention time of 5.5 min on a μBondapak C_{18} (10 μm) analytical HPLC column (Fig. 1).

Quantitative elution of both creatinine and 4-AMP from the cation-exchange resin was not effected through the use of 3.0 ml of 0.5 *M* sodium actetate as suggested by Lim et al. [5]. We found that 4.0 ml of 1 *M* sodium acetate afforded the quantitative elution of both creatinine and 4-AMP from the resin.

Using this HPLC method, we obtained creatinine concentrations from 24-hour urines (47 samples) that were an average of 14% lower than the creatinine concentrations obtained from these same 24-hour urine samples using the alkaline picrate method [7]. This result was not unexpected due to all of the variables that influence the results of the alkaline picrate method [2]. In a recent communication Chiou et al [4], using a cation-exchange HPLC column for the separation of creatinine from interfering plasma components and ultraviolet detection, reported plasma creatinine concentrations that were an average of 14.55% lower than the creatinine concentrations obtained from the same plasma samples using a SAM-6 Auto Analyzer. The automated creatinine analyzer utilizes the alkaline picrate method [2, 3].

Table I shows the accuracy of this HPLC method and the alkaline picrate method in the analysis of aqueous creatinine solutions. Both methods have about the same accuracy. It can be concluded that endogenous "quasi-creatinine" [2] compounds in the urine are responsible for the elevated urinary creatinine concentrations obtained by the alkaline picrate method.

The linearity of the HPLC method was investigated over the range 0.40—4.00 mg creatinine per ml urine, with a correlation coefficient of 0.970. Because of the relatively high urine creatinine concentrations (mg/ml range) the lower limit of sensitivity of this procedure was not determined. Repetitive analysis (nine times) of a single urine sample from a normal, healthy male afforded an average creatinine/4-AMP peak height ratio of 0.82 ± 0.06 (mean ± S.D.). The standard deviation is about 7% of the mean.

TABLE I

A COMPARISON OF THE HPLC METHOD AND THE ALKALINE PICRATE METHOD USING AQUEOUS CREATININE SOLUTIONS

Actual concentration (mg/ml)*	HPLC creatinine (mg/ml)**	Alkaline picrate creatinine (mg/ml)**
1.71	1.68	1.53
1.20	1.14	1.11
1.37	1.50	1.30
1.03	1.00	1.00

*Aqueous creatinine solutions prepared by colleague and analyzed "blind" by both procedures.
**Duplicate determinations with the average value recorded.

When 24-hour urinary indoleacetic acid (IAA) concentrations, expressed as mg IAA per mg creatinine, for normal control patients and for schizophrenic patients were based upon creatinine concentrations obtained by the alkaline picrate method [7], the IAA concentration for schizophrenic patients was significantly higher, at the 0.007 level, than for the control patients. When this same ratio was based upon creatinine values obtained from the HPLC procedure, the IAA concentration for schizophrenic patients was significantly higher (at the 0.001 level) than for the control patients [8].

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