Micromethod for Determination of Creatinine in Biological Fluids by High-Performance Liquid Chromatography

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We describe a procedure for the rapid and specific measurement of creatinine, in which it is separated from other compounds in serum or urine by paired-ion chromatography and is quantified by measuring its absorbance at 200 nm. The procedure can be done on as little as 10 µl of serum. Between-day precision studies for concentrations of 13 and 62 mg/liter yielded coefficients of variation of 6.9 and 2.2%, respectively. Analytical recovery of various amounts of creatinine added to plasma exceeded 95% in all cases. The proposed procedure was compared with the continuous-flow procedure by analyzing a series of serum and urine specimens by both methods. There was excellent agreement for urine specimens, but with serum the results by the present method were significantly ($P<0.001$) lower.

In 1886 Jaffé (1) described the reaction between creatinine and alkaline picrate to yield a red chromogen, and in 1914 Folin (2) first used it for measuring creatinine concentrations in biological fluids. Although the lack of specificity has long been realized, the Jaffé reaction is an important part of the clinical chemists' armamentarium, and currently is the basis for all of the commonly used methods of analysis for creatinine. Most attempts to improve test specificity have depended on the separation of creatinine from the pseudocreatinine chromogens before the color reaction and have included the use of cation-exchange resins (3), Lloyd's reagent (4), or dialysis (5). Improvements have also been sought through modification of the conditions under which the final chromogen has been produced as, for example, by acidification of the final reaction mixture to take advantage of the fact that the true creatinine chromogen is less resistant to acidification than are the pseudochromogens (6).

In the early 1970's Cook (7), Larsen (8), and others suggested that in biological fluids the initial rate of the Jaffé reaction is proportional to the concentration of creatinine and that this rate differs significantly from the rate of reaction of pseudo-creatinine chromogens in the same system. This led to the hope that the specificity of the test could be improved by choosing a suitable time "window" during which the reaction rate would be monitored. Unfortunately, this hope has only been partly realized and it is now known that reaction-rate procedures are subject to several interferences (9).

Recently, more specific procedures for the analysis of creatinine have been described, including the enzymatic methods of Wahlefeld et al. (10) and Szasz and Börner (11) and the method proposed by Brown et al. (12), in which high-performance liquid chromatography (HPLC) is used to separate creatinine from the pseudochromogens before the Jaffé color development.

The HPLC method we describe differs in two important aspects from the method of Brown et al. (12): we use paired-ion chromatography\(^1\) to separate creatinine from other compounds in serum or urine, and the creatinine is quantified solely by measuring its absorbance at 200 nm, thereby circumventing use of the Jaffé reaction. The procedure affords excellent specificity, requires as little as 10 µl of serum (although we routinely use 25 µl), and takes less than 5 min per analysis.

Materials and Methods

**Materials**

**Apparatus.** Throughout these studies we used a high-performance liquid chromatograph Series 2/2 (Perkin-Elmer Corp., Norwalk, Conn. 06856) and a 4 mm × 30 cm μ-Bondapak C-18 column mounted in a temperature control block (both from Waters Associates, Inc., Milford, Mass. 07157). The detector used was a Perkin-Elmer Model LC-55 variable wavelength spectrophotometer attached to a Perkin-Elmer/Coleman 550 recorder.

**Reagents.** We obtained methanol from Burdick and Jackson Laboratories, Inc., Muskegon, Mich. 49442, and 3-hydroxybutyric acid, lactic acid, pyruvic acid, acetoacetic acid, and creatine from Sigma Chemical Co., St. Louis, Mo. 63178. Chemicals were also obtained from

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\(^1\) Paired-ion chromatography allows for the simultaneous assay of acids, bases, and neutral compounds, by use of the reversed-phase technique. The retention time of compounds of interest can be relatively easily controlled and modified by changing the concentration of the appropriate counterion (e.g., sodium lauryl sulfate) in the mobile phase.
the Fisher Scientific Co., Chemical Manufacturing Division, Fairlawn, N.J. 07410 (creatinine, urea, uric acid), from Calbiochem, Los Angeles, Calif. 90054 (α-ketoglutaric acid), and from BDH Chemicals Ltd., Poole, England (sodium lauryl sulfate and amino acids). Reagent A consists of potassium phosphate buffer (20 mmol/liter, pH 5.1) containing 80 mg of sodium lauryl sulfate per liter. Reagent B is methanol. Control sera, Versatol A Alternate and Validate, were obtained from General Diagnostics, Division of the Warner-Lambert Co., Morris Plains, N.J. 07950.

Procedure

Serum or plasma. Add 75 μl of methanol to 25 μl of serum or plasma in a 6 × 50 mm glass test tube. Vortex-mix the mixture for 30 s, then centrifuge for 1 min. Inject exactly 6 μl of the supernate directly into the chromatograph and elute with a 76/24 (by vol) mixture of A/B. The flow rate is 2.3 ml/min, the column temperature 30 °C. The detector sensitivity setting should be 0.02 A full scale. Approximate pressure at the head of the column is 13.8 MPa (2000 psi).

The creatinine peak on the chromatogram is identified by its retention time, and the amount is measured by comparing the peak height obtained for the sample with a standard curve obtained by injecting known amounts of creatinine standard into the system. Figure 1 shows the results obtained for the analysis of a typical serum sample.

Urine. Dilute 30-fold and then treat exactly as above. Figure 2 shows the results obtained for the analysis of a typical urine sample.

Because it takes about 4 h for the column to stabilize, we continually pump the eluting solution through the column (although at rates of only 0.1 ml/min when it is not being used for creatinine analysis). The reagent cost so incurred is far outweighed by the ready availability of an emergency procedure for creatinine.

Results and Discussion

Analytical Variables

Detector wavelength. Maximum absorption of creatinine in the buffer described is at 200 nm, and this wavelength is used because it provides more sensitivity than the 230–240 nm region (Figure 3). Ideally, a bi-chromatic approach would be optimal, with the column eluate being continuously monitored at 200 and 230 nm.
Conceivably this could increase specificity, as creatinine has a known absorbance ratio at these wavelengths and the presence of an interfering compound would in all probability alter the ratio, thereby alerting the operator to the problem.

Column temperature. We chose to use a temperature of 30 °C; the viscosity of the eluting solution increases significantly at lower temperatures, resulting in undesirably high column pressures (up to 27 MPa).

Composition of eluent. Quantitation of creatinine is possible with the buffer pH in the range 3.2–5.1. However, a pH at the upper end of the range is preferred because the optimum temperature for the analysis decreases with decreasing pH, resulting in high column back-pressures. We also tried varying the concentration of sodium lauryl sulfate (the paired-ion reagent). Higher concentrations of this reagent increase the strength of the cation-exchange column and therefore increase retention times with a resulting broadening of peaks. The solvent ratio was varied over a wide range; a ratio of 76/24 provides good separation of creatinine from interfering compounds while maintaining a short (about 160 s) retention time and good sensitivity.

Method of Calculation

By injecting known amounts of creatinine (in methanol) into the system, a standard curve for absorbance vs. amount of creatinine is obtained. We recommend that this standard curve should be obtained daily, although its slope does not change appreciably from day to day. It is linear to a creatinine concentration of 40 mg/liter under the conditions described.

Precision

We assessed the precision of the method on a day-to-day basis by repeated analysis of Versatol A Alternate and Validate containing different concentrations of creatinine. At concentrations of 13 and 62 mg/liter, the method yielded coefficients of variation of 6.9 and 2.2%, respectively. Corresponding coefficients of variation with the routine continuous-flow procedure in our hospital are 7.7 and 1.8%.

Accuracy

Recovery. Portions of a plasma pool containing 6 mg/liter of creatinine were augmented to achieve final concentrations of 23 and 35 mg/liter. Analytical recovery was 95.3% (n = 10, CV = 2.1%) and 98.0% (n = 10, CV = 2.9%), respectively.

Method comparison. The proposed procedure was compared with the continuous-flow (AutoAnalyzer) procedure by analyzing a series of patients' specimens (serum and urine) by both procedures. As recommended by Barnett and Youlden (13), no more than five serum or urine samples were analyzed on any one day. Statistical analysis of the data obtained for serum (Figure 4) revealed that the present procedure gave results that were significantly lower than those obtained by the continuous-flow method (P < 0.001).

These results confirm those recently published by others (11, 12, 14), all of whom found the routine continuous-flow procedure to give considerably higher results than the more specific procedures developed in their laboratories. Perhaps of far greater significance is the large interindividual variation in non-creatinine chromogens, a factor previously shown in the excellent study carried out by Doolan et al. (14) and now confirmed by our studies. Of what potential significance is this? Of the 48 patients studied here, three had creatinine results by HPLC that were ≤50% of the value obtained with the AutoAnalyzer: 4, 4, and 9 mg/liter as compared with 10, 12, and 18 mg/liter, respectively. Depending on the clinical situation, such differences may lead to changes in a patient's classification as normal or abnormal, or to changes in a patient's treatment as, for example, while following the course of a patient after a renal transplant.

It is well accepted that urine contains less non-creatinine Jaffé-positive chromogen than serum. Our results (Figure 5) for 32 patients' samples analyzed both by the HPLC and continuous-flow procedures showed excellent agreement, an observation that is in harmony with this fact.
Interference Studies

Two groups of compounds were tested and found not to interfere with the procedure described. Group A includes compounds that by their nature might have interfered with the procedure; Group B includes some non-creatinine Jaffé-positive compounds which are of importance because they often are present in clinical samples.


Group B: Acetoacetic acid, pyruvic acid, α-ketoglu-taric acid, methyldopa (15) and cephaloridine (16).

Thus, if the presence of any of the compounds in Group B is suspected or known, the present procedure, but not a Jaffé-based procedure, will provide a reliable measurement of creatinine.

We thank Dr. D. M. Goldberg for his help in the preparation of this manuscript and acknowledge the generous support of Perkin-Elmer Corporation in providing the HPLC equipment.

References