Serum Creatinine Determination by High Performance Liquid Chromatography and Five Automated Chemistry Analyzers

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ABSTRACT

A sensitive and specific procedure is described for the determination of serum creatinine by reverse phase high performance liquid chromatography. The method involves sample pretreatment with a cation exchange resin followed by the isocratic separation of creatinine and an external standard which are detected by their absorbances at 254 nm. The analytical recovery of creatinine from serum was 99.8 ± 4.9 percent using this method. The between-day coefficients of variation for creatinine concentrations of 0.83 and 8.63 mg per dl were 3.07 percent and 3.2 percent, respectively. Results obtained with this reference method were compared to results obtained with five automated chemistry analyzers. The results showed that while the comparison methods correlated well with the reference method (r > 0.98 in all cases), those comparison methods based on the Jaffe reaction showed negative biases. In some cases, these biases were highly statistically significant. The difference between paired results obtained with some of the automated Jaffe-based assays exceeded 0.20 mg per dl in a substantial number of specimens with creatinine concentration in the range of 0.5 to 2.3 mg per dl.

The measurement of acetoacetate concentrations of specimens submitted to our laboratory for serum creatinine assays showed that interference by this substance with certain Jaffe-based assays could result in clinically significant false elevations in serum creatinine concentrations.

Introduction

The reaction of creatinine with alkaline sodium picrate was first described by Jaffe in 1886. Since that time, this color reaction has been used with numerous modifications for the determination of serum creatinine. As might be expected
in the case of a well-studied, widely-used assay, a number of substances that interfere with this reaction have been identified.\textsuperscript{2,3,14,16}

The nonspecificity of the Jaffe reaction for serum creatinine is dealt with in several ways. The first of these involves pretreatment of serum specimens with adsorbents, organic solvents, protein precipitants or dialysis.\textsuperscript{10} However, these techniques have met with variable success. With the exception of dialysis, none is suitable for use with automated instrumentation. A second and more popular approach to increasing specificity in serum creatinine assays is to use kinetic versions of the Jaffe reaction. These kinetic assays aim to differentiate chromogen formation by creatinine from that due to interfering substances by differences in their reaction rates.\textsuperscript{7} A third approach to a more specific assay for creatinine is to abandon the Jaffe reaction entirely in favor of creatinine methods based on the use of enzymes as reagents\textsuperscript{10} or methods based on high performance liquid chromatography (HPLC).\textsuperscript{1,4,9,11,12}

The existence of a variety of assays for serum creatinine as well as the variability among the automated kinetic Jaffe assays with respect to their precise assay conditions\textsuperscript{3} suggests the possibility of intra-method and inter-instrument bias in creatinine results. The purpose of this study was: (1) to develop a sensitive, specific, and accurate HPLC assay for serum creatinine and (2) to use this assay as a reference method for the evaluation of the creatinine test on five automated chemistry analyzers.

Materials and Methods

Serum Creatinine by HPLC

The following HPLC assay for serum creatinine was developed by the present authors based on the previous work of Lim et al.\textsuperscript{9} The first part of the procedure is a sample pretreatment designed to isolate creatinine from other serum constituents. The basis of this pretreatment step has already been described.\textsuperscript{1}

Mini-columns containing 100 mg of Dowex AG 50W X-12 were poured in 2.5M NaOH and washed with 2.0 ml of H\textsubscript{2}O and 4.0 ml of adsorption buffer (0.04 M citric acid, 0.02 M dibasic sodium phosphate, pH 3.0). Exactly 0.5 ml of sample was mixed with 5.0 ml of adsorption buffer, and 5.0 ml of the mixture was applied to the minicolumn. Each column was washed with 4.0 ml H\textsubscript{2}O\textsubscript{2}, and the adsorbed creatinine was then eluted with 3.0 ml of 0.5 M sodium acetate, pH 8.6. One hundred \(\mu\)l of a solution of 0.58 mM allopurinol in 0.5 M sodium acetate was added to each eluent as an external standard.\textsuperscript{12}

Twenty \(\mu\)l of each eluent was injected onto a 4 mm \(\times\) 30 cm column of 10 \(\mu\)M silica particles to which a unimolecular layer of octadecyltrichlorosilane was bonded.* High pressure liquid chromatography was carried out using 0.01 M ammonium acetate as the mobile phase at a flow rate of 1.0 ml per min. Creatinine and allopurinol were detected by their absorbances at 254 nm. The creatinine concentration of a sample was calculated by comparing its peak height ratio (creatinine peak height per allopurinol peak height) to those of a series of creatinine standards† that were carried through the entire procedure.

The analytical recovery of creatinine by this method was assessed by comparing the peak height ratios of extracted and unextracted aqueous creatinine standards and by adding pure creatinine in increments of 0.3 to 18 mg per dl to pooled human serum. The average recoveries are based on results from tests

\* u-Bondapak C\textsubscript{18}, Waters Associates, Milford, MA.
\† New England Reagent Laboratory, East Providence, RI.
performed in duplicate on two separate occasions. The precision of the method was established by assaying commercial control sera on 10 occasions over a one month period.

**Comparison Methods**

Creatinine results determined by HPLC were compared with the following automated methods using a split-sample protocol: Dupont ACA III (ACA),§ Technicon SMAC (SMAC),§ Beckman ASTRA (ASTRA),‖ Multistat (MCA),¶ and EKTACHEM 400 (EKTA).** Unless specified otherwise, each automated instrument was operated according to the manufacturer's instructions using the calibrators and reagents that they supplied. The ASTRA instrument was also operated using reagents manufactured by Nobel Scientific (ASTRAN).††

A series of aqueous standards were assayed as samples on each instrument (except EKTA) to check for systematic bias that might occur as a result of differences between calibrators.

**Clinical Specimens**

The 72 serum specimens used in the method comparison study were obtained from blood drawn in red-topped vacutainers and submitted to the laboratory for biochemical profiling. Specimens with glucose values greater than 140 mg per dl were excluded from this group. The creatinine concentrations of the specimens as determined by HPLC ranged from 0.4 to 23.2 mg per dl (mean, 4.5 mg per dl, median, 1.0 mg per dl). Serum was separated into aliquots for each of the comparison methods and frozen. Since it was not possible to assay each specimen by all of the methods, comparison results for the MCA, ASTRAN and EKTA methods are based on subsets of the group of 72. Acetoacetate was measured on specimens sent to the laboratory for creatinine determination. Specimens from known diabetics were excluded from this group.

**Interference Studies**

The responses of the reference and test methods to several known interfering substances were tested using pooled serum spiked with the substances of interest in the concentrations indicated in the Results section. Acetoacetate concentrations in clinical specimens were determined by an enzymatic method.8

**Statistical Evaluation**

Method comparison data were analyzed by linear regression techniques.5 Since the differences between paired results for individual specimens assayed by various combinations of reference and comparison method were not normally distributed according to the Kolmogorov-Smirnov Test,15 the significance of these differences was tested using the Sign Test. Differences that were significant at the 0.05 level by the Sign Test were further evaluated using Wilcoxin's Test.15

**Results**

**HPLC Assay for Serum Creatinine**

In figure 1 is shown the elution profile obtained when a serum specimen with a creatinine concentration of 3 mg per dl was extracted and chromatographed as described in the Methods section. Cre-
Figure 1. Analysis of serum creatinine by HPLC. A serum specimen with a creatinine concentration of 3 mg per dl was applied to a cation exchange mini column which was eluted as described in Methods. Twenty μl of the eluent was chromatographed on a 4 mm × 30 cm column of uBondapak C18. Mobile phase: 0.01 M ammonium acetate, flow rate: 1.0 ml per min, detection: absorbance at 254 nm, 0.01 AUFS. Peak 1: Creatinine 0.81 n mol), Peak 2: Allopurinol (0.39 n mol). Specimens with creatinine concentrations greater than 6 or 12 mg per dl were chromatographed as described except that the detector sensitivity was decreased to 0.02 AUFS or 0.05 AUFS, respectively.

Creatinine and the external standard had retention times of approximately 4.6 and 9.5 minutes, respectively, and peak heights of approximately 115 and 97 mm, respectively. The two peaks eluting before creatinine have not been identified. However, they were also observed when aqueous creatinine standards were analyzed.

The mean recovery of creatinine from the cation exchange mini-columns was 100.3 percent (sd, 6.0 percent; range 94 to 106 percent) for a series of aqueous standards with creatinine concentrations of 1.0 to 10.0 mg per dl. The analytical recovery of creatinine from serum specimens with concentrations in the range of 0.3 to 18 mg per dl averaged 99.8 percent (sd, 4.9 percent; range 95 to 109 percent).

The overall standard deviations for the assay over a one month period for serum controls with creatinine concentrations of 0.83 and 8.63 mg per dl were 0.025 and 0.267 mg per dl respectively. The retention times for creatinine and the internal standard were constant for a period of at least six months, and the HPLC column showed no signs of decreased performance after more than 250 analyses.

Comparison of Methods

No systematic bias between the various automated Jaffe-based methods was detected when a series of aqueous creatinine standards was analyzed. The results of the split-sample correlation experiment in which patients' sera were assayed by HPLC and different automated methods are shown in table I. Results from the automated methods correlated well with those of the reference method. However, in all cases, the automated Jaffe-based methods gave results that were higher than those obtained by HPLC. The intermethod differences observed in the HPLC/SMAC, HPLC/
ASTRA, and HPLC/MCA comparisons were highly statistically significant.

To provide a more practical example of the magnitude of the intermethod differences that might be encountered when serum creatinine is determined using two different automated analyzers, ASTRA creatinine results were compared with ACA and SMAC creatinine results (table II). For both ranges of creatinine concentrations examined (Group 1 and Group 2, table II), the differences observed in the ASTRA/ACA and ASTRA/SMAC comparisons were highly statistically significant. These intermethod differences frequently exceeded 0.35 mg per dl for Group 1 specimens and 0.20 mg per dl for Group 2 specimens.

INTERFERENCE

The HPLC assay for serum creatinine was not affected by glucose (100 to 1500 mg per dl), ascorbate (0.5 to 4.0 mg per dl), hemoglobin (27 to 320 mg per dl), or acetoacetate (0.5 to 5.0 mM). These potential interfering substances did not affect the automated methods except in the following cases: glucose at 1500 mg per dl caused a 0.1 mg per dl increase in creatinine as measured by the ASTRA and ASTRA-N methods; acetoacetate caused severe positive interference in the ASTRA, ASTRA-N, and SMAC methods. In all three methods, acetoacetate increased the apparent creatinine concentration in a linear fashion: ASTRA and ASTRA-N, 0.24 mg per dl increase per mmol per liter acetoacetate; SMAC, 0.18 mg per dl increase per mmol per liter acetoacetate. When acetoacetate was
measured in serum from a random sampling of 30 non-diabetic inpatients on whom serum creatinines were ordered, a mean of $0.42 \pm 0.33$ mmol per liter was found. Serum acetoacetate was greater than 0.5 mmol per liter in eight patients and greater than 1.0 mmol per liter in three patients.

Discussion

The increased popularity of HPLC as an analytical tool has led to the establishment of serum creatinine assays based on ion exchange,\textsuperscript{1,4} paired ion,\textsuperscript{11} and reverse phase\textsuperscript{9,12} techniques. All of the HPLC methods appear to offer adequate sensitivity, precision, and specificity for creatinine. The methods differ in the complexity of the sample pretreatment and the complexity of the analytical system. The ion exchange method of Brown et al\textsuperscript{4} does not require sample pretreatment prior to assay; however, creatinine in the column effluent is detected by the Jaffe reaction using a home-made microanalyzer. The ion exchange method of Ambrose et al\textsuperscript{1} requires preliminary deproteinization of the samples with trichloroacetic acid followed by gradient elution of creatinine from the HPLC column, with detection by absorbance at 234 nm. The paired ion method of Soldin and Hill\textsuperscript{11} features a simple pretreatment step consisting of deproteinization with methanol followed by isocratic HPLC with detection by absorbance at 200 nm. This method, although extremely sensitive, is less precise than the others and yields more complicated chromatograms. The reverse phase method of Spierto et al\textsuperscript{12} involves deproteinization by ultrafiltration followed by isocratic HPLC with detection at 236 nm. An internal standard is included in this procedure resulting in improved precision. However, the chromatograms obtained using this method are complex owing to the limited sample pretreatment, — suggesting the potential for interference by matrix constituents in some samples.

The combination of sample pretreatment with cation exchange resins followed by reverse phase liquid chromatography was originally used without an external standard to purify creatinine from biological fluids prior to analysis by gas chromatography/mass spectrometry. Creatinine isolated from serum using this technique was found to be homogeneous,\textsuperscript{9} suggesting that the procedure could serve as a reference method for serum creatinine.

The features of the method as presented here include: (1) a high analytical recovery of creatinine, (2) the use of an external standard, (3) good precision, (4) high specificity, and (5) stable chromatographic conditions. Moreover, the sample pretreatment and the conditions of the HPLC separation are such that the test can be performed inexpensively using the most basic of HPLC equipment. While the method as presented here would not lend itself to routine use in the determination of serum creatinine, it has been found by the present authors to be useful as a reference method.

The results of our method comparison showed that serum creatinine results obtained with five automated chemistry analyzers correlated with those obtained by our HPLC reference method. Despite the excellent correlations, all of the test methods except the EKTA, were negatively biased relative to HPLC. Given the high analytical recovery and the specificity of this reference method, it would appear that the biases might be due, in part, to interference by non-creatinine chromogens in the Jaffe-based test methods. Differences in the responses of the test methods to creatinine is another possible cause of the biases observed by us. Both proportional and constant biases were reported in four dif-
different Jaffe-based assays in a previous study in which the reference method consisted of ion-exchange sample pretreatment prior to creatinine determination by the Jaffe reaction. The intermethod variability observed by us could be important when more than one instrument is used for serum creatinine determination in a laboratory. In a previous study, a decrease in serum creatinine of 0.35 mg per dl (from an initial value of 2.6 mg per dl) was identified as the average medically significant change by a group of 63 specialists in internal medicine. Based on our data, an inter-method difference of this magnitude would not be uncommon. Such differences would be especially important when the serum creatinine is in the normal range where small changes may be indicative of a significant change in a patient’s glomerular filtration rate.

The potential for the misinterpretation of changes in serum creatinine concentrations as a result of intermethod biases is further emphasized by the differential effects of interfering substances on various methods. While interference by some of the most common Jaffe interferents has been eliminated in the automated methods used by us, acetoacetate severely interfered with two of the methods. The magnitude of the interference was such that the increase in the “apparent” creatinine concentration would be small for acetoacetate concentrations in the normal range (<0.2 mM). In physiological and pathological hyperketonemic states, acetoacetate interference may be substantial depending on the “true” creatinine concentration of the sample and the ratio of acetoacetate (which interferes with the assay) to beta-hydroxybutyrate (which does not interfere). In extreme hyperketonemic states such as diabetic ketoacidosis, the serum acetoacetate concentration may range from 1.5 to 10.0 mM. In a previous study, the average blood acetoacetate concentration in 18 patients upon initial presentation with diabetic ketoacidosis was 3.03 ± 1.0 mM (range, 1.5 to 5.4). It is notable that two out of 30 specimens selected at random from a hospitalized, non-diabetic population had acetoacetate concentrations sufficient to cause a spurious increase of 0.2 to 0.4 mg per dl in the creatinine result as determined by the SMAC or the ASTRA.

References

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