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Note

Determination of creatine in serum by ion-pair high-performance liquid chromatography with fluorometric detection

LAWRENCE E. WEBB

Travenol Laboratories, Inc., P.O. Box 490, Round Lake, IL 60073 (U.S.A.)

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The determination of creatine in serum is a useful test for the diagnosis of muscle disease. A sensitive serum creatine assay is also needed to test the hypothesis that this compound is produced in inadequate amounts under conditions of inadequate nutrition or compromised hepatic function [1].

Colorimetric methods for the determination of serum creatine generally lack sensitivity and specificity. The widely used indirect colorimetric assay, based on the Jaffe reaction, determines creatine by the difference in creatinine before and after conversion of the creatine into creatinine. Method errors are large because this difference is small compared with the total creatinine and because the Jaffe reaction is not specific [2]. Arginine and other guanidino compounds interfere in the α-napththol—diacetyl colorimetric procedure [3]. Coupled enzyme procedures [4, 5] also lack the sensitivity required for serum determinations and are subject to background reactions and interference by bilirubin [6].

Several high-performance liquid chromatographic (HPLC) ion-exchange methods have been developed for the determination of guanidino compounds in physiological fluids, and these methods have the required selectivity for creatine. Increased sensitivity is achieved by post-column derivatization with phenanthrenequinone, benzoin or ninhydrin followed by fluorescence detection. Of the several systems of this type [7—9], the one using ninhydrin as the fluorescence reagent is by far the most sensitive for creatine [9]. However, the chromatographic separation requires six different eluent solutions. A reversed-phase ion-pairing method using two C_{18} columns in series has been reported [10], but the phenanthrenequinone post-column reaction used does not detect creatine.

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This paper describes the combination of the ninhydrin post-column detection procedure with a simple isocratic reversed-phase ion-pairing procedure to produce a sensitive and specific assay for creatine.

EXPERIMENTAL

Chemicals

Creatine monohydrate, creatinine, guanidinosuccinic acid, and guanidinopropionic acid were all obtained from Sigma (St. Louis, MO, U.S.A.). Guanidinoacetic acid and L-arginine hydrochloride were from Aldrich (Milwaukee, WI, U.S.A.). Monochloroacetic acid and sodium hydroxide were from Mallinckrodt (St. Louis, MO, U.S.A.), ninhydrin from Sargent-Welch (Skokie, IL, U.S.A.), and sodium octyl sulfate from Kodak (Rochester, NY, U.S.A.).

Solutions

The mobile phase was 0.05 *M* monochloroacetic acid, buffered to pH 3.0 with sodium hydroxide and containing 10 mg/l sodium octyl sulfate. The mobile phase, 0.75 *M* sodium hydroxide solution, and 0.6% ninhydrin solution were each passed through a 0.22-μm filter before use.

Standards

Working standards (1, 2, 5, 8 and 10 mg/l) were prepared by diluting a 1000 mg/l stock standard with water. The stock standard was prepared from the anhydrous creatine obtained by drying creatine monohydrate at 110°C for 3 h. Standards were used within five days of preparation [11].

Chromatographic system

The mobile phase was pumped at 0.7 ml/min by an Isco Model 2300 pump (Lincoln, NE, U.S.A.). Creatine was separated on a Zorbax C_{18}, 5-μm, reversed-phase column (25 cm × 4.6 mm I.D.) at ambient temperature (DuPont, Wilmington, DE, U.S.A.).

The post-column reactor was that described for guanidines [9], with the temperature and geometry of the reaction coil adapted for creatine. The column effluent was first mixed with 0.75 *M* sodium hydroxide in a T-junction, and then mixed with 0.6% ninhydrin in a Kel-F® Tee. The effluent—reagent mixture then entered the post-column reaction coil, which was heated in a water bath to 40°C, the optimum temperature for creatine [9]. The reaction coil was prepared by winding 30 m of 0.3 mm I.D. (30-gauge) Teflon® tubing around a cylinder. One end of this tubing was pulled through a short piece of flanged 1.5 mm O.D. tubing and then connected to the Kel-F Tee with a 1.5-mm tubing fitting [12]. The other end of the 0.3 mm I.D. reaction coil tubing was connected to the detector with a Minitight nut (Upchurch, Oak Harbor, WA, U.S.A.).

A Perkin-Elmer Series 3B dual-pump system (Norwalk, CT, U.S.A.) delivered the alkaline solution and nonhydrin solution at flow-rates of 0.6 and 0.4 ml/min, respectively.

Detection was performed with a Perkin-Elmer Model LC-10 filter

fluorometer. A mercury phosphor conversion lamp and filter provided excitation at 360 nm. A 418—700 nm emission filter was used. Data reduction was done by a 3357 Laboratory Automation System (Hewlett Packard, Avondale, PA, U.S.A.).

Procedure

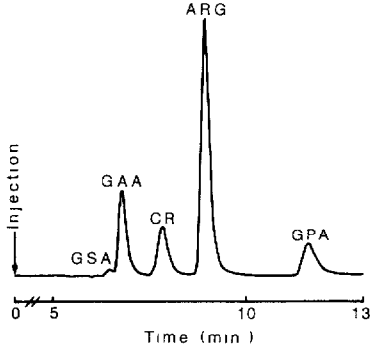
Samples were prepared by mixing 200 μl of serum standard with 100 μl of 10% trichloroacetic acid. After centrifugation at 10 000 *g* for 5 min, the supernates were transferred to a microfilter (Bioanalytical Systems, West Lafayette, IN, U.S.A.). The microfilters were centrifuged at 1000 *g* for 3 min and 20 μl of filtrate were injected onto the HPLC column. Smaller injection volumes were used if the sample concentration was greater than 10 mg/l. Creatine concentrations were calculated from peak areas using linear regression analysis of the standards for calibration.

RESULTS

A typical separation of a standard solution of creatine and closely related guanidino compounds is shown in Fig. 1. The mobile phase composition was optimized at pH 3.0, in accordance with the previous observation that some guanidino compounds require a low pH for effective separation by reversed-phase ion-pair chromatography [10].

Fig. 2 shows a typical chromatogram from a plasma sample. In addition to the large creatine and arginine peaks, a small, late-eluting peak was observed at 20 min in the chromatograms from some serum samples. Peaks from other guanidino compounds were well separated from the creatine peak and were generally not detectable at the injection volumes used. For example, creatinine elutes near guanidinopropionic acid and requires a concentration corresponding to 100 mg/l in serum to be detected.

The fluorescence response, expressed as peak area, was linear for 25—1270 pmol creatine injected (equivalent to 0.2—12.5 mg/l in serum). Above this



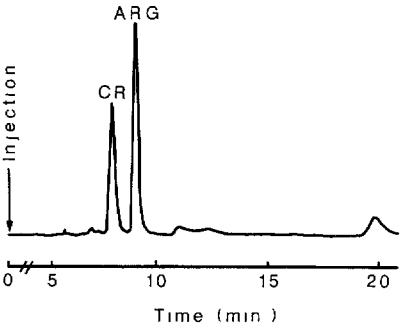


Fig. 1. Chromatogram of a standard mixture of guanidino compounds. Peaks: GSA = guanidinosuccinic acid; GAA = guanidinoacetic acid; CR = creatine; ARG = arginine; GPA = guanidinopropionic acid. Amounts injected were 0.4 nmol of GSA, 2.3 nmol of GAA, 0.2 nmol of CR, 5 nmol of ARG, and 1.5 nmol of GPA.

Fig. 2. Chromatogram of a serum sample containing 2.4 mg/l creatine. Peaks: CR = creatine; ARG = arginine.

range, peak areas occasionally showed a slight positive deviation from linearity. The 1—10 mg/l range was used for standard curves. The limit of detection (signal-to-noise ratio 2:1) was 5 pmol creatine injected.

To determine the analytical recovery, 5.4 mg/l creatine was added to aliquots of a normal serum pool containing 3.6 mg/l creatine. The mean recovery was 105.2% (standard deviation = 3.6%, n = 7).

For two serum pools with mean creatine concentrations of 3.31 and 9.04 mg/l, the intra-assay coefficients of variation were 2.53 and 2.28%, respectively (n = 7). For an additional estimate of precision, a normal plasma pool ($\bar{x}$ = 1.64 mg/l), which was stored at —70°C, was assayed one to four times on each of ten different days during eleven months. The overall coefficient of variation was 5.8% (n = 21). The intra-assay and inter-assay coefficients of variation were 2.54 and 6.01%, respectively, as estimated by analysis of variance.

The creatine concentrations measured in sera from fourteen healthy adult laboratory workers ranged from 1.6 to 8.7 mg/l with a mean (and standard deviation) of 3.9 (2.6) mg/l. These values are within the reference ranges determined by other methods [2, 8].

DISCUSSION

The high sensitivity of ninhydrin to creatine makes this reagent well suited to creatine measurement. I optimized the sensitivity for creatine relative to that of other guanidino compounds by using a 40°C post-column reaction temperature [9]. The low detection limit of the creatine method suggests that it could easily be adapted to small sample volumes (< 100 μl). Body fluids such as urine or uremic sera, which contain a relatively high amount of creatine, could be assayed by reduction of sample or injection volumes.

The present method is relatively simple to implement. The isocratic ion-pair technique simplifies the chromatography, and materials for the post-column system are economical and readily available. Post-column derivatization with benzoin or phenanthrenequinone requires a strong base and an organic solvent; the alkaline ninhydrin system has the advantages of simplicity and relatively mild reagents that are readily soluble in water. The combination of chromatography and post-column detection used for the creatine assay should also prove useful for the determination of other guanidino compounds.

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