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# Determination of creatinine in serum by high-performance liquid chromatography: a comparison of three ion-exchange methods

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## SUMMARY

Three ion-exchange high-performance liquid chromatographic methods for the determination of creatinine in serum have been compared. In method 1 a strong cation exchanger was used. In method 2 a reversed-phase column was given strong cation-exchange properties by the addition of N-methyloleoyl taurate to the mobile phase. In method 3 a weak cation exchanger was used. Elution was with a pH gradient in methods 1 and 2, and isocratic elution was used in method 3. The imprecision was similar for the three methods and varied between 0.9 and 2.5% as studied within-day and between 1.4 and 3.2% from day-to-day. The lowest coefficient of variation was obtained around the upper reference limit. Analytical recoveries were quantitative for the three methods. The method with N-methyloleoyl taurate showed no advantages over the conventional strong cation exchanger. With the weak cation exchanger no interferences were detected from compounds investigated, but with the strong cation exchanger a slight interference was obtained with uric acid. We prefer the weak cation-exchange method because of its simplicity, higher throughput and absence of interference from hitherto tested compounds.

## INTRODUCTION

Determination of serum creatinine is used world-wide for the estimation of renal function, both as a screening or diagnostic method and for detection of short-term changes in patients with known renal disease. For this purpose a large number of determinations have to be performed, and the analytical methods have to be automated. The most popular methods for serum creatinine are based on the well known alkaline picrate reaction described by Jaffé [1] more

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than 100 years ago. Folin [2] applied this reaction to the determination of creatinine in deproteinized serum, and although numerous modifications have been described, these method variants are more or less susceptible to a number of interferents [3]. Therefore other methods have been developed (see the recent review by Spencer [4]). Most popular among methods other than the Jaffé method are different kinds of enzymic methods [5–10]. However, like the Jaffé method [11], some enzymic methods are biased by high bilirubin concentration in the sample [12,13], others by hemolysis [5] or by certain drugs [14,15].

Obviously, there is a need for a reliable comparison method to be used when interferents are suspected in routine creatinine analysis. The isotope dilution mass spectrometric methods [16–18] have been proposed as reference or definite methods, but they require expensive equipment and are not suited to the ordinary laboratory.

During the past 20 years high-performance liquid chromatography (HPLC) technology has developed quite dramatically, and such methods for creatinine determination have been in use since 1977, either conventionally [19], e.g. with on-line detection of creatinine in the effluent, or as a prepurification step before gas chromatography–mass spectrometry (GC–MS) [16]. Although HPLC for serum creatinine is easy to perform, only ca. 30 methods have been published. These include cation-exchange [19–24], normal-phase [25] and reversed-phase (RP) [26–35] modes of liquid chromatography. In the latter case ion-pair reagents have also been employed [27,33].

Although these methods are all attractive we thought that the ion-exchange methods should be most suitable. Therefore we have now evaluated three different cation-exchange HPLC procedures in order to find out which one would be most suitable for our purposes, e.g. to measure serum creatinine accurately in samples suspected to contain compounds interfering in our routine methods and for comparison purposes in the evaluation of new routine methods.

## EXPERIMENTAL

### *Reagents*

All chemicals used in the procedure were reagent grade. Creatinine standard was obtained from Sigma (St. Louis, MO, U.S.A.). A stock solution, 0.265 m*M*, was prepared in 20 m*M* hydrochloric acid and was used for preparations of appropriate diluted standard solutions. The creatinine iminohydrolase, EC 3.5.4.21 (creatinine deiminase), xanthine derivatives and creatine used for interference studies were also from Sigma, and the compound N-methyloleoyl taurate [$C_{17}H_{33}$–CO–N($CH_3$)–$CH_2$–$CH_2$–$SO_3^-$] was obtained from GAF (Wayne, NJ, U.S.A.).

*Apparatus*

The automated HPLC instrumentation consisted of a Model 2249 gradient pump, a Model 2141 variable-wavelength monitor and a Model 2221 integrator (LKB, Bromma, Sweden). The samples were injected by a Model 460 autosampler from Kontron Instruments (Tegimenta, Switzerland).

Creatinine was detected at 234 nm because the uncharged molecule shows an absorption maximum at this wavelength [21]. The flow-rate was 1.0 ml/min.

*HPLC columns*

The material in the strong cation-exchange column was Nucleosil 5SA packed by Jones Chromatography in 150 mm × 4.6 mm I.D. stainless-steel columns.

As the reversed-phase $C_{18}$ column, to which N-methyloleoyl taurate was adsorbed, we used a 250 mm × 4.6 mm I.D. LiChrosorb RP-18 column (7 μm) from Merck (Darmstadt, F.R.G.).

The weak cation-exchange column was a 150 mm × 4.6 mm I.D. column from the Bio-Rad (Richmond, CA, U.S.A.) urinary metanephrine kit, Cat. No. 195-6001.

*Procedure*

*Protein precipitation and blank experiments.* To 500-μl aliquots of aqueous standard or serum specimen, 500 μl of trichloroacetic acid (0.6 mol/l) were added. The mixture was vigorously agitated and centrifuged at 2000 *g* for 10 min. The supernatants were decanted and 20 μl were injected into the liquid chromatograph. The samples were prepared and chromatographed both before and after treatment with creatinine iminohydrolase. We used this enzyme to investigate whether the assumed creatinine peak was due solely to the presence of creatinine or if interfering serum compounds could contribute to the peak. Therefore we added either 50 μl of Tris buffer (0.1 *M*, pH 7.6) or 50 μl of Tris buffer containing 20 kU/l creatinine iminohydrolase to the 500-μl serum aliquots, mixed and incubated at 37°C for 60 min before protein precipitation.

A calibration curve of peak height versus creatinine concentration was used to quantify the unknown samples.

*Chromatography.* Chromatography was performed on three different cation-exchange columns.

The strong cation-exchange column (method 1) had covalently bound $RSO_3^-$ as active ionic groups. Elution buffer A contained 25 m*M* lithium acetate (pH 4.45) mixed with methanol (92:8, v/v) and elution buffer B consisted of 100 m*M* lithium acetate (pH 7.25) also mixed with methanol (92:8, v/v). After injection, mobile phase A was pumped through the column for 2 min. Then a linear gradient was created during 1 min until 100% of mobile phase B was obtained. This eluent was used for a further 6 min, and the system was then reverted to 100% mobile phase A (1 min). After equilibration for 10 min the system was ready for another injection.

For method 2 N-methyloleoyl taurate was absorbed onto an RP column by including 200 mg/l Igepon T-77 in the elution buffer and equilibrating the column for at least 24 h. The modified column acquires strong cation-exchange properties [36] and has also $RSO_3^-$ as active ionic groups.

The creatinine was eluted in the same way as for the strong cation-exchange column with slightly different elution buffers. The composition of elution buffer A was 25 m*M* lithium acetate (pH 4.68) with 8% methanol and 200 mg/l Igepon T-77, and elution buffer B contained 75 m*M* lithium acetate (pH 7.1) with 12.5% methanol and 200 mg/l Igepon. Gradient elution was identical with that of method 1.

The weak cation-exchange column (method 3) had covalently bound $RCOO^-$ as active ionic groups. This makes the column unable to completely bind the creatinine at the low pH values used in the other applications. At pH 4.8, however, the creatinine is retained long enough to be separated from interfering peaks. Gradient elution was not necessary and the elution buffer used was 15 m*M* lithium acetate (pH 4.8)–methanol (95:5, v/v).

## RESULTS

### *Chromatography*

A representative chromatogram obtained with system 1 is shown in Fig. 1A. Lithium acetate buffer has lower absorbance at high pH values, but increased concentration of lithium acetate in buffer B compensates for this difference.

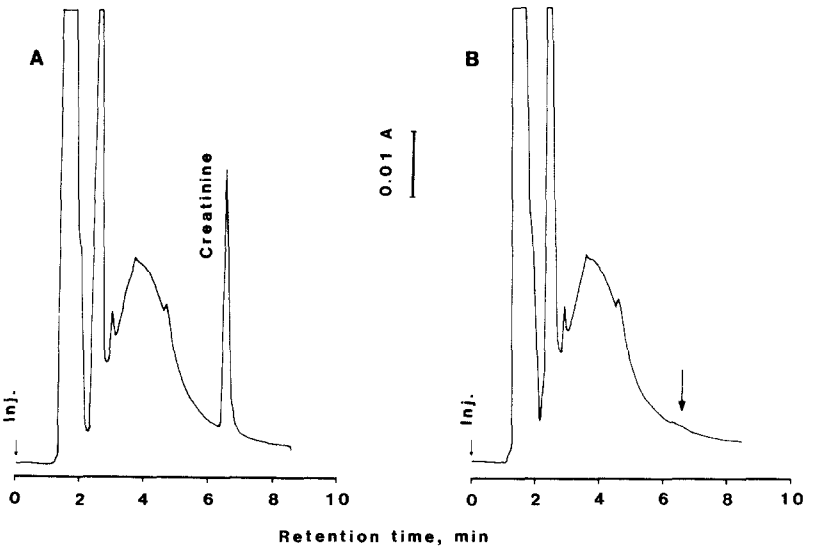


Fig. 1. Chromatogram of serum containing 92.5 $\mu M$ creatinine before (A) and after (B) treatment with creatinine iminohydrolase. Chromatography was with the strong cation exchanger.

After the initial trichloroacetate peak there was a broad peak, presumably due to the shift in pH and acetate concentration. After the creatinine has been eluted (8 min) the column has to be equilibrated with buffer A for another 12 min. This means that a sample injection can be performed every 20 min. Fig. 1B shows the results when the serum was incubated with creatinine iminohydrolase before analysis. The entire creatinine peak has disappeared, and no interfering peak from other serum components can be seen. The creatinine iminohydrolase releases $NH_3$ from creatinine to give N-methylhydantoin, which is not detected in the chromatography.

The results with the serum sample chromatographed on the Igepon T-77 column are shown in Fig. 2. Together with the broad gradient effect, there are four additional peaks, one before and three after the creatinine peak. These peaks are an effect of the pH gradient on the Igepon column and can also be seen in the standard chromatogram. Because the gradient and equilibration times are the same as before, one sample can be processed every 20 min. By enzyme conversion of creatinine we found also this system to be free from interfering peaks (Fig. 2B).

A sample chromatogram from the weak cation-exchange column is shown in Fig. 3. Because only one elution buffer has to be used, samples can be injected every 9 min, twice as often as with the other two systems.

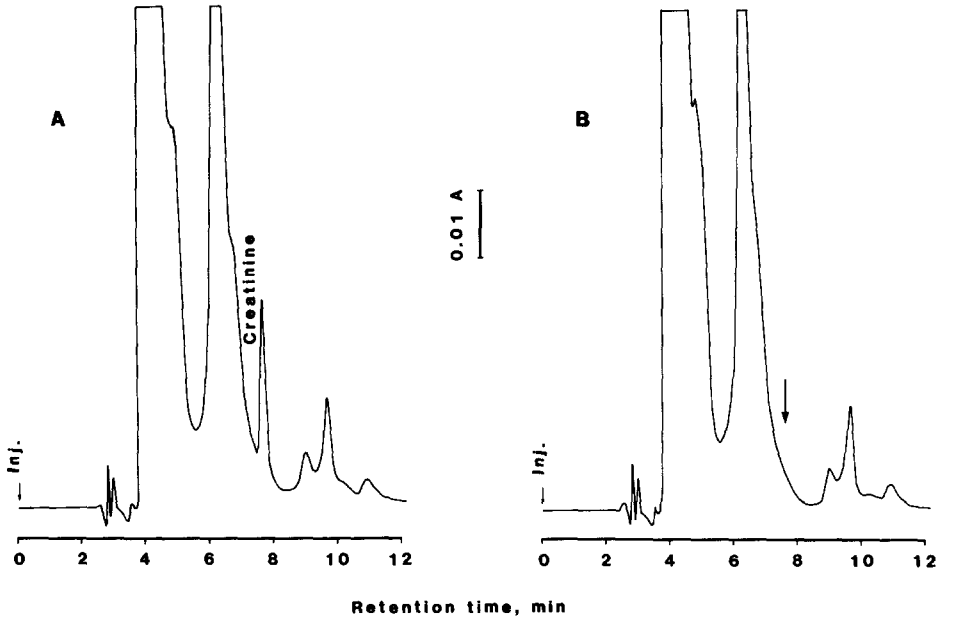


Fig. 2. Chromatography of same serum as in Fig. 1 with Igepon T-77 in the mobile phase.

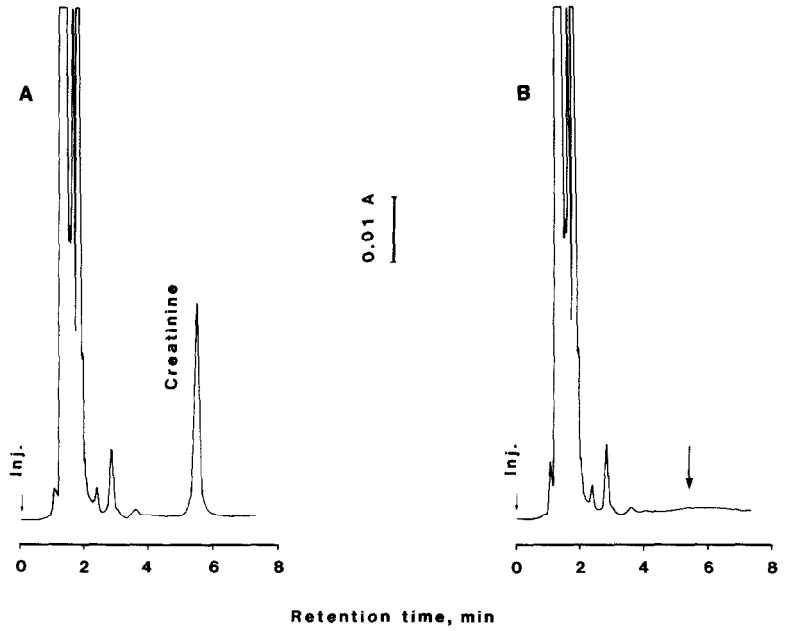


Fig. 3. Chromatography of the same serum as in Fig. 1 on a weak cation exchanger.

*Linearity of standard curves*

The standard curves for creatinine obtained with the three methods were linear with an injected amount of 0–40 nmol. This corresponds to creatinine concentrations of 0–8000 $\mu M$ when analysed according to the procedure for serum.

*Precision and detection limit*

Analytical imprecision was estimated from duplicate analysis of samples with different concentrations. We used fifteen to twenty samples for each of the ranges 40–80 $\mu M$, 80–120 $\mu M$ and 120–700 $\mu M$. The within-day coefficients of variation (C.V.) at different levels (mean $\pm$S.D. within parenthesis) were 1.2% ($60 \pm 0.7\ \mu M$), 0.9% ($94 \pm 0.8\ \mu M$) and 1.8% ($384 \pm 6.9\ \mu M$) for method 1. With method 2 the C.V. were 1.7, 1.4 and 2.5%, and with method 3 the C.V. were 1.4, 1.2 and 2.5% at the same mean levels. Day-to-day imprecision was determined from single analyses repeated on two consecutive days with the same samples. The C.V. were 1.7, 1.5 and 2.7% (method 1), 2.2, 1.8 and 3.1% (method 2) and 2.0, 1.4 and 3.2% (method 3). For estimation of total (day-to-day) analytical imprecision we determined the concentration of one sample during ten days with all three systems. The C.V. were 1.5, 1.4 and 1.7% for methods 1, 2 and 3, respectively, at a creatinine level of 86 $\mu M$.

With system 3 we tried to evaluate the detection limit in terms of detection

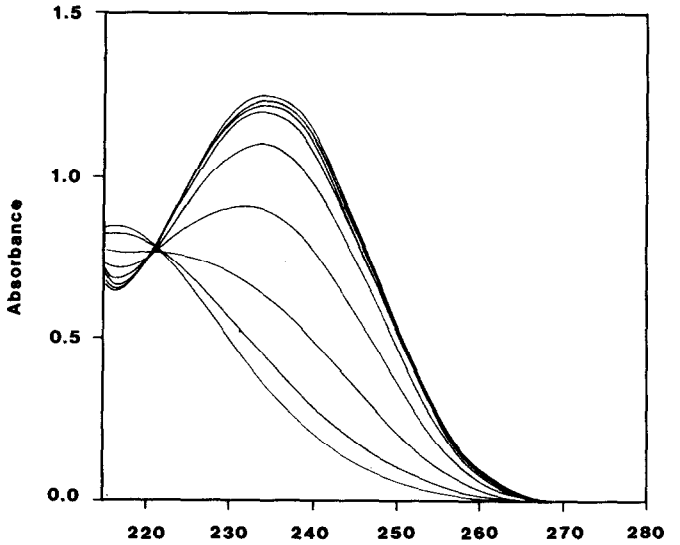


Fig. 4. Absorbance spectrum for creatinine at pH 3.5 (lowest absorbance at 234 nm), 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0 and 7.8 (highest absorbance at 234 nm). Measurements were done with a Uvicon 930 (Roche) spectrophotometer. Creatinine (200 $\mu M$) was dissolved in a solution of 15 $M$ lithium acetate–methanol (9:1, v/v), and recordings were subtracted by the absorbance of the dissolving solution (pH 4.8).

from noise. By this method the detection limit was estimated to be 0.5 $\mu M$ (three times the noise), which is quite satisfactory. Fig. 4 shows the absorbance spectrum of creatinine at different pH values. Maximum absorbance at 234 nm was obtained at pH > 7.0, and at pH 4.8 ca. two thirds of that value was obtained. Therefore, the detector signal was higher for creatinine on elution by the gradient systems (higher pH and higher UV absorption at 234 nm), which would result in a lower detection limit. However, because of the elution of creatinine together with non-specific gradient effects, small changes due to creatinine were concealed and the detection limit was about the same as with system 3.

*Recovery experiments*

We added creatinine to ten serum samples with different creatinine concentrations to increase their concentration by 98 $\mu M$ and reanalysed the samples by all the three methods. The recoveries obtained were $101.2 \pm 3.8$, $103.4 \pm 4.3$ and $102.7 \pm 4.0\%$ with methods 1, 2 and 3, respectively.

*Interference studies*

We investigated possible interference from uric acid, caffeine, xanthine, hypoxanthine, 1-methylxanthine, 9-methylxanthine, theophylline, theobromine, propoxyphylline and etophylline by injecting 20 $\mu l$ of these compounds at con-

centrations of 300–500 $\mu M$. None of these compounds interfered with the weak cation exchanger (method 3). With the strong cation exchanger (method 1) uric acid, xanthine, 1-methylxanthine and 9-methylxanthine gave peaks close to or at the same retention time as creatinine. A uric acid concentration of 500 $\mu M$ gave a peak corresponding to 2.9 $\mu M$ creatinine. With the three other xanthine derivatives (xanthine, 1-methylxanthine and 9-methylxanthine) the peak height was less than 10% of that from creatinine, on a molar basis. Creatine did not interfere in any of the methods.

## DISCUSSION

To our knowledge a weak cation exchanger has not previously been used for serum creatinine HPLC determination. Such columns cannot be utilized at very low pH values because of the weak acid properties of the R–COOH group. However, at its $pK_a$ value close to 5.0 [21,37] or slightly below, creatinine was retained strongly enough for separation. Gradient elution was not necessary, which had the effect that the analytical capacity was twice that of systems 1 and 2.

The chromatographic results with the strong cation exchanger were as expected. We speculated, however, that use of the cation-exchange reagent N-methyloleoyl taurate for semi-irreversible loading of the $C_{18}$ reversed-phase material to give the matrix ion-exchange properties [36] might improve the separation. However, the chromatograms were quite similar to those obtained with the strong cation-exchange column, and gradient elution had to be utilized. For the present we do not think this method has any advantages over ordinary strong cation exchangers.

Based on the physiological day-to-day intra-individual serum creatinine variation of 4.2–4.4% (expressed as a C.V.), and the recommendation that the tolerable analytical variability should be less than half of the biological variation, Spencer [4] defined the analytical imprecision goal for serum creatinine to be 2.2%. Except for high values the methods described here are within this target. The three methods have their best performance at the levels around the upper reference limit with C.V. from 1.4 to 1.8%, and the analytical recovery was quantitative.

Different HPLC methods should be evaluated from the authors' own data for imprecision and recovery. A literature search revealed that out of four published ion-exchange methods all had C.V. values below 2.2% [19,21,23,24], and when reported, recovery was quantitative [21,22,24]. With reversed-phase chromatography there are only two papers with reported within-day imprecision better than 2.2% [28,30], but in eight methods the C.V. was lower than 4% [28–33]. With only a few reversed-phase methods has the analytical recovery been reported close to 100% with reasonable precision [31,33]. We conclude that, as far as precision and analytical recovery are concerned, our three

methods compare well with published ion-exchange HPLC methods. Such methods seem to have better precision and analytical recovery than reversed-phase methods.

Most HPLC methods for human serum creatinine rely on the UV absorption of creatinine for detection. Thus detection has been performed at 196 [34], 200 [27], 210 [33], 215 [20,22,23], 220 [28,30], 235 [32], 236 [29,35] and 254 [21,25,26,28,31] nm, and only exceptionally has detection been after post-column reaction [19,38]. From the pH-dependent UV absorbance curve for creatinine it seems that low wavelengths should be used when eluting at low pH if high sensitivity is required. This increases the risk of interference from other UV-absorbing compounds, but may be necessary if the creatinine analysis is to be combined with analysis of urea [34], for example. With elution at higher pH the absorbance of the uncharged molecule at higher wavelengths (maximum ca. 235 nm) could be utilized.

Our results show that preincubation of serum with the enzyme creatinine iminohydrolase completely destroyed the creatinine in serum, and chromatography of such treated serum gave no deflection at the retention time of creatinine. This indicates that normally no interfering compounds will be found in serum for either of the methods. However, we added xanthine derivatives to serum and found that, with method 1, uric acid, xanthine, 1-methylxanthine and 9-methylxanthine all gave small peaks near that of creatinine. The uric acid concentration is ca. 120–360 $\mu M$ in healthy subjects [39]. This would give an overestimation of creatinine by 0.7–2.0 $\mu M$, and with hyperuricemia up to 1000 $\mu M$ [40] an overestimation of 5.8 $\mu M$ may be obtained. The normal xanthine concentration in serum is $1.4 \pm 0.7$ $\mu M$ [39] and is barely detected. Plasma xanthine concentrations of 13.2–39.6 $\mu M$ have been observed in patients with xanthine oxidase deficiency [39]. Such concentrations of xanthine would give peaks corresponding to creatinine concentrations of less than 4 $\mu M$. With method 1 we recently found a hitherto unidentified peak interfering with the creatinine. On re-analysis of the sample by method 3, however, the extra peak was well separated from the creatinine peak.

Although the three HPLC methods evaluated have similar precision and quantitative analytical recovery, we prefer the weak cation-exchange method because of its simplicity, higher throughput and absence of interference from hitherto tested compounds.

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