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Assessment of a Selected Method for Creatinine with Special Emphasis on Bilirubin Interference

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Summary: An HPLC-based method for the determination of creatinine was evaluated, with emphasis on interference by bilirubin.

The method differed from other procedures, in that deproteinization was by ultrafiltration instead of precipitation with trichloroacetic acid. This allows a rapid, direct analysis by HPLC with very few experimental steps.

Analytical results showed linearity up to 5000 μmol/l and an average analytical recovery of 100%.

Within-day imprecision was 0.8–1.4%, and day-to-day imprecision was 0.5–2.4%.

Introduction

It has been known for years that the routine determination of creatinine in serum can present laboratory workers with serious problems.

The traditionally used methods are all based on the well known *Jaffé* reaction (1). In spite of the many modifications of this method, interference by one or more substances still exists. A comprehensive list is given by *Young* et al. (2).

We met this problem in our laboratory after the introduction of the Technicon Chem 1 analyser. Clinicians criticized the value of some creatinine results, and most of the specimens in question turned out to be icteric. Bilirubin, the most troublesome interferent in the creatinine determination, is elevated (> 20 μmol/l) in about 16% of our daily workload. It therefore appeared expedient to study the state of the art of the Chem 1 analyser with respect to interference by bilirubin with the creatinine determination. We have already drawn attention to this interference in a previous publication (3).

It was clear that in order to answer this question a reliable method was needed. From the literature it is known that high-performance liquid chromatography (HPLC) can be suitable analytical method.

High-performance liquid chromatography for the determination of creatinine in serum has been described, using both reversed-phase (5–8) and ion exchange systems (9–12).

However, almost all techniques described have a low throughput, either due to long column preconditioning or to gradient elution.

Furthermore the use of trichloroacetic acid, used in a number of techniques as a deproteinizing agent, is not free of risks. The risks are inclusion of creatinine and conversion of creatine to creatinine (4).

We therefore considered ultrafiltration as an independent means of deproteinization (13). Deproteinization of serum by ultrafiltration and subsequent direct analysis of the aqueous supernatant could offer a true advantage over the established methods that use trichloroacetic acid.

Materials and Methods

Materials

Reagents

All chemicals were at least reagent grade. Creatinine (> 99%), bilirubin, trichloroacetic acid (20%), acetic acid (100%), hydrochloric acid (37%), dimethylsulphoxide, sodium carbonate, methanol Lichrosolv and acetic acid, were obtained from Merck, Darmstadt, Germany. The concentration of trichloroacetic acid used was 0.6 mol/l (10%).

Lithium acetate dihydrate, MicroSelect, was obtained from Fluka BioChemika AG Buchs, Switzerland.

Creatinine iminohydrolase (EC 3.5.4.21) used for interference studies was obtained from Sigma, St Louis, MO, U.S.A.

Water was purified with the Milli-Q water system from Millipore, Molsheim, France.

A stock solution of 10 mmol/l creatinine was prepared in 20 mmol/l hydrochloric acid and was used to make seven aqueous standard solutions covering the range 50–1000 µmol/l.

The serum samples were stored for up to one month at −80 °C until further use.

Standard reference material 909 from the National Institute of Standards and Technology, U.S.A. was used together with two levels (Kontrollogen-L and -LP) control sera from Behringwerke A.G., Marburg, Germany, to evaluate the HPLC method.

Furthermore, six reference sera (A, B, C, D, E, F) from the Deutsche Gesellschaft für Klinische Chemie, Zentrale Referenzinstitution were used as control material. In all sera the creatinine determination was performed by isotope dilution-mass spectrometry and by a validated reference method.

Methods

Ultrafiltration

The disposable Centrifree Micropartition System from Amicon used in a fixed-angle rotor (JA-21 Beckman) provides an ultrafiltrate which is almost free from protein after centrifugation at 1000 *g*.

In the removal of protein from a serum sample by ultrafiltration, there is a decrease of the total sample volume due to the removal of proteins.

A factor of 0.9456 is used to adjust for this change in volume according to *Weast* (15).

Sample preparation

A serum sample (100–300 µl) was centrifuged for 20 minutes and 10 µl ultrafiltrate was directly injected into the HPLC system.

Blank sample preparation

Serum (500 µl) was mixed with 50 µl Tris buffer containing 20 kU/l creatinine iminohydrolase and incubated in a closed tube for 60 minutes at 37 °C.

Bilirubin interference

The protocol worked out by the Société Française de Biologie Clinique was used to study interference by bilirubin (14).

High performance liquid chromatography (HPLC)

The HPLC system from Spectra Physics consisted of a SP8800 HPLC pump, a SP8780 autosampler, a SP8790 column heater, a SP8450 UV detector and a SP4290 integrator. The HPLC procedure is essentially according to *Kagedal & Olsson* (11).

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

The elution buffer was lithium acetate 15 mmol/l. The pH was adjusted to 4.80 with acetic acid and filtered (0.45 µm cellulose acetate/nitrate membrane, Millipore, Molsheim, France) before use. The eluent was a mixture of lithium acetate – methanol (95 + 5 by vol.), and the flow rate was 1.0 ml/min.

Because there was no dilution of the sample with trichloroacetic acid as in the original procedure, we preferred to reduce the injection volume from 20 to 10 µl in order to minimize peak broadening. The column temperature was raised to 40 °C, in order to diminish peak tailing. The absorbance was monitored at 234 nm, and calibration was performed with aqueous external standards.

Results

Preliminary experiments, performed exactly according to the procedure of *Kagedal & Olssen* with aqueous standard material, occasionally showed a second peak or tailing peaks. We suspected trichloroacetic acid, because reduction of the amount of trichloroacetic acid more or less solved this problem. We decided to study this phenomenon at a later stage and to circumvent the problem by applying the Amicon Centrifree Micropartition System, because this system had worked very well in initial experiments. This system provides an almost protein- and bilirubin-free ultrafiltrate and requires only 100 µl of serum.

Because of this modification we had to re-examine the precision, accuracy, linearity, sensitivity and recovery of the method.

Precision

Analytical imprecision was calculated from two commercially available human control sera with creatinine levels of 123.5 µmol/l (Kontrollogen L) and 293 µmol/l (Kontrollogen LP). Within-day imprecision was determined by six replicate analyses of Kontrollogen L and LP.

Between day imprecision was assessed using the same controls, divided into aliquots and stored at −20 °C. On 10 separate occasions an aliquot of each level was thawed and analysed. The results are shown in table 1.

Accuracy

To evaluate the accuracy of the HPLC method, we analysed seven sera, the creatinine values of which had been determined by isotope dilutions-mass spectrometry or by a reference method (see tab. 2).

Tab. 1. Within- and between-day imprecision of creatinine measurements.

	Mean µmol/l)	SD (µmol/l)	CV (%)
Within-day (n = 6)	121	1.7	1.4
	296	2.2	0.8
Between-day (n = 10)	121	1.9	1.6
	293	3.7	1.3

Tab. 2. Accuracy of creatinine measurements by HPLC.

Pool	Reference value[a] (µmol/l)	Mean[b] (µmol/l)	SD (µmol/l)	CV (%)	Recovery[c] (%)
A	538	544.1	12.8	2.4	101.1
B	155	161.0	2.5	1.6	103.9
C	221	218.2	4.1	1.9	98.7
D	109	110.1	2.6	2.4	101.0
E	593	607.0	12.0	2.0	102.4
F	120	121.1	1.2	1.0	100.9
SRM 909	151.3	151.2	0.8	0.5	99.9
Kontrollogen L	123.5	120.8	0.9	0.7	97.8
Kontrollogen LP	293.0	297.9	2.9	1.0	101.6

[a] Values determined by the reference method used by the 'Deutsche Gesellschaft für Klinische Chemie', except for S.R.M. 909.
[b] mean values are calculated from n = 4 except for Kontrollogen L and LP, where n = 10.
[c] Percent of the reference value.

For all sera the mean concentration of creatinine measured by HPLC was within 97.8% to 103.9% of the stated value.

Linearity

We used a calibration curve prepared from aqueous creatinine standards covering the range 0–1000 µmol/l creatinine. A representative calibration curve is shown in figure 1. The method proved to be linear up to 5000 µmol/l, using an extended calibration set of 1500, 2000, 3000, 5000 µmol/l.

Sensitivity

The sensitivity in terms of the differentiation of a peak from background noise was estimated to be 0.25 µmol/l (three times the noise).

Recovery

Recovery of creatinine from the ultrafiltration system was checked by means of three aqueous creatinine

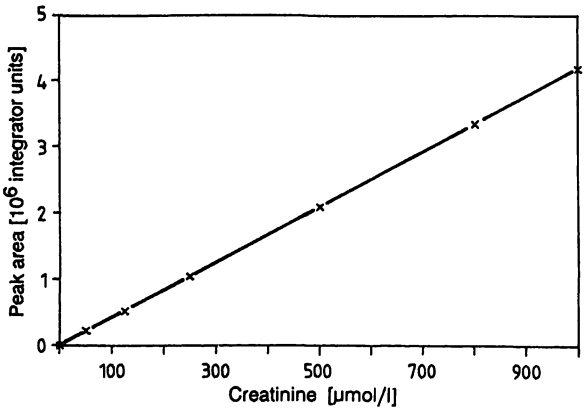

Fig. 1. Calibration curve prepared from aqueous creatinine standards; all points represent the mean of triplicate injections. $y = 4398x + 700$, $S_x = 0.8$, $S_y = 700$, $n = 18$, $r^2 = 0.999999$.

standards (125, 500 and 1000 µmol/l). Furthermore we added an aqueous creatinine standard (100 µmol/l) to ten serum samples with different creatinine concentrations and the same standard to ten serum samples with a creatinine level of about 90 µmol/l (see tab. 3).

To be absolutely sure that the ultrafiltrate represents the true creatinine concentration of the sample, we also used the standard-addition technique to estimate the creatinine concentration of the two calibrators, Kontrollogen L and Kontrollogen LP, and the S.R.M. 909 of the National Institute of Standards and Technology.

Creatinine was added to a serum sample at a concentration of zero, once and twice the estimated creatinine concentration. Four HPLC analyses were performed on each sample.

The average area of these four analyses was used to calculate the linear regression line through these three points. The recovery of the additions was found to be 99.8% ± 0.5%.

The results are shown in table 4.

Interference by bilirubin

Conjugated and unconjugated bilirubin were investigated separately as possible sources of interference. The conjugated samples were taken from our daily workload, by measuring the total bilirubin level as well as the conjugated fraction with the Technicon Chem-1. The selected samples had a total bilirubin concentration of > 100 µmol/l, range 102–564 µmol/l, mean 210 µmol/l, the conjugated fraction being elevated, range 10–279 µmol/l, mean 117 µmol/l.

Tab. 3. Analytical recovery of creatinine after ultrafiltration.
a) aqueous standards;
b) 10 serum samples with a creatinine content of approximately 90 µmol/l;
c) 10 serum samples with a creatinine content ranging from 65 to 305 µmol/l.

	Creatinine		
	added (µmol/l)	found (µmol/l)	recovery (%)
a. *Aqueous standards*			
	120	119.3	99.4
	500	497.5	99.5
	1000	1000.9	100.1
b. *Serum, low creatinine*			
1	–	81	
	100	183	102
2	–	89	
	100	188	99
2	–	99	
	100	198	99
4	–	93	
	100	193	100
5	–	85	
	100	185	100
6	–	83	
	100	185	102
7	–	82	
	100	183	101
8	–	72	
	100	173	101
9	–	75	
	100	175	100
10	–	71	
	100	170	99
(n = 10)			$\bar{x}$ = 100.3%
c. *Serum, high creatinine*			
11	–	65	
	100	165	100
12	–	167	
	100	268	101
13	–	342	
	100	442	100
14	–	375	
	100	474	99
15	–	172	
	100	273	101
16	–	137	
	100	237	100
17	–	249	
	100	349	100
18	–	201	
	100	300	99
19	–	166	
	100	264	98
20	–	305	
	100	405	100
(n = 10)			$\bar{x}$ = 99.8%

Tab. 4. Estimation of the creatinine concentration in triplicate of Kontrollogen L and LP, and S. R. M. 909 with standard addition to eliminate volume changes due to filtration.

Pool	Reference value[a] (µmol/l)	Run 1 (µmol/l)	Run 2 (µmol/l)	Run 3 (µmol/l)
SRM 909	151.3	151.1	151.6	152.4
Kontrollogen L	123.5	120.7	120.2	118.8
Kontrollogen LP	293.0	298.7	299.5	292.9

[a] Values determined by the reference method used by the 'Deutsche Gesellschaft für Klinische Chemie', except for S. R. M. 909.

serum was incubated with creatinine iminohydrolase, and an ultrafiltrate was also analysed with the HPLC.

In none of 100 samples did we find interfering peaks due to bilirubin or other substances. An example is given in figure 2, a serum sample with a total bilirubin concentration of 385 µmol/l.

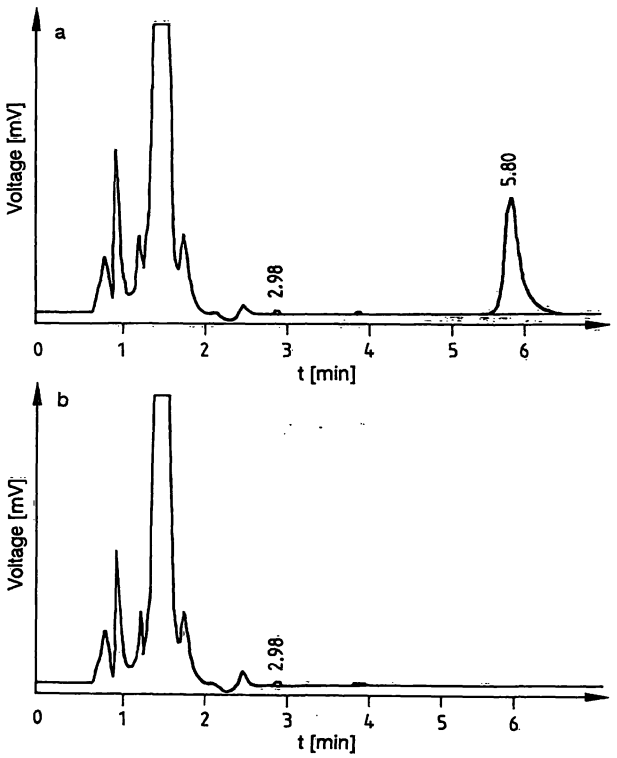

Fig. 2. Chromatogram of a serum sample with an elevated bilirubin concentration of 385 µmol/l and a creatinine concentration of 85 µmol/ before (a) and after (b) treatment with creatinine iminohydrolase; the peak at 5.80 minutes is creatinine, attenuation is 32 (2^5). 1000 mV = full scale

Conjugated bilirubin

We analysed approximately 100 samples with the HPLC method and with the Chem 1 analyser, each sample being analysed twice. First an ultrafiltrate from the native serum was used to measure the creatinine concentration, next an aliquot of the same

Non-conjugated bilirubin

We investigated bilirubin added to serum, and added to an albumin/creatinine mixture, according to the CERMAB protocol (14). The creatinine concentrations in serum were 78, 179 and 269 µmol/l and in the albumin solution 89, 223 and 445 µmol/l. The

bilirubin concentrations 7, 30, 60, 110, 250, 360, 420 μmol/l in all dilutions.

The results revealed no interference from unconjugated bilirubin, as shown in figure 3.

Apart from these samples, we studied samples from neonates. These samples (n = 15) were incubated with creatinine iminohydrolase. Again we found no interfering peak in any chromatogram.

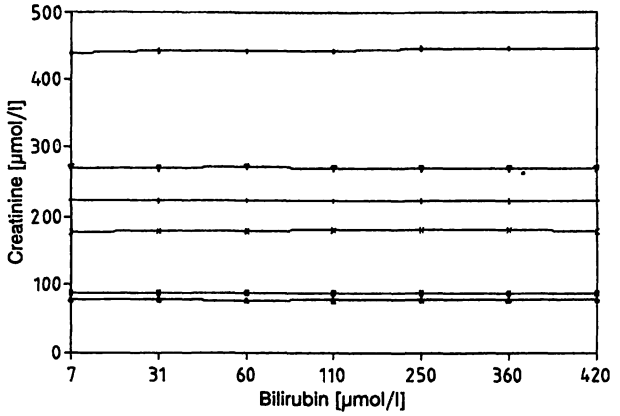

Fig. 3. The interferogram of bilirubin. Serum samples (creatinine 78 (▲), 179 (x) and 269 (▼) μmol/l) and albumin samples (creatinine 89 (■), 223 (|) and 445 (♦) μmol/l) did not show any interference.

Discussion

In his review of the estimation of creatinine (16) *Spencer* mentioned the importance of the effect of bilirubin in kinetic *Jaffé* procedures. Furthermore, he said that HPLC methods are relatively quick for single estimations and that the between-day precision could be compared to most *Jaffé* procedures.

The weak cation exchange procedure of *Kagedal & Olssen* (11), has introduced a new era of creatinine estimations. Interference studies showed no interference by uric acid, caffeïne, xanthine and several drugs, but bilirubin was not investigated. Because of the importance of creatinine concentration as a clinical parameter and because of the negative interference of elevated bilirubin concentrations we wondered whether the HPLC procedure could serve as an alternative for our routine method in emergency cases. However, the use of trichloroacetic acid also possibly causes interference, so we circumvented this problem by using an ultrafiltration step. A second advantage of the replacement of trichloroacetic acid by the Amicon ultrafiltration system is the elimination of all dilution steps, and in our opinion this is the reason that our precision and recovery are somewhat better than reported by *Kagedal & Olsson*. Accuracy also proved to be good. Retention times for creatinine were reproducible and unaffected by creatinine concentration and injection volume.

Treatment with creatinine iminohydrolase proved that the peak with a retention time of 5.80 minutes was solely due to the presence of creatinine.

Elevated bilirubin levels did not once show interference with any sample after treatment with creatinine iminohydrolase.

Furthermore, co-elution of an interfering substance was not observed in any of the examined "normal" samples used in the initial experiments.

In conclusion, the use of HPLC in combination with a weak cation exchanger to determine creatinine in serum is in our view a very fast, easy to use and accurate back-up system for patient sera with elevated bilirubin levels. In general we feel that our method meets the standard of a candidate reference method. We prefer the use of the standard addition technique instead of applying a protein correlation factor. At the moment we are comparing both modifications, together with a determination of the water content (17).

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