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Validation of the Ultrafiltration Technique for Creatinine Analysis by HPLC: A Comparison with Direct Serum Injection

By *L. Zwang* and *B. G. Blijenberg*

Department of Clinical Chemistry, Academic Hospital Rotterdam-Dijkzigt, The Netherlands

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Summary: The determination of creatinine by HPLC was performed by direct injection of serum onto the column, and after ultrafiltration of the sample, and the results were compared. A modified weak cation exchange column was used for HPLC. This eliminates the protein displacement effect and the *Gibbs-Donnan* effect, thus permitting a rapid direct analysis. Three of the four ultrafiltration methods gave almost identical analytical results. Regression analyses of the results from direct serum injection versus ultrafiltration showed a bias of 5% on the slope $y = 1.050 \cdot x_{\text{pure serum}} + 1.856$, which was in our view solely due to the volume displacement effect of the proteins; correction of each data point by a protein-dependent correction factor resulted in an almost perfect regression line.

Introduction

The value of high-performance chromatography (HPLC) as an analytical method for the determination of creatinine in serum, depends on its design and application. Several publications report the use of this technique as a (candidate) reference method, or a selected method (1–5).

In others, HPLC is simply used to measure creatinine concentrations in serum or urine (6–10).

In the latter case, a rapid throughput and thus a minimal number of experimental steps is desired. In a reference technique, however, time is not as important as precision and accuracy. In both applications, it is necessary to remove proteins, either by acid precipitation or by ultrafiltration (12, 13) and irrespective of the chosen technique, the volume displacement effect of the removed proteins must be taken into account in the interpretation of the results.

The technique as described by us (5) is an easy-to-use and accurate back-up system for the comparison of large sets of creatinine samples. Because of the workload we felt the need to improve the absolute speed of our HPLC system (6 samples/hour).

We therefore considered two options for decreasing the operation time by a factor 2 or 3:

a. use of a shorter column

b. increased flow.

In addition, we wished to investigate the problem of accucary by studying the ultrafiltration step in more detail. We therefore compared the ultrafiltration technique with a HPLC method developed by us, which does not involve deproteinization, and we also compared different ultrafiltration units.

Materials and Methods

Materials

Reagents

All chemicals were essentially the same as in our previous study (5).

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

Kontrollogen L and Kontrollogen LP control sera from Behringwerke A. G., Marburg, Germany, together with three reference sera (A, B, C) from the Deutsche Gesellschaft für Klinische Chemie (Zentrale Referenzinstitution) were used as con-

trol material. In all sera, the creatinine was determined by isotope dilution-mass spectrometry and by a validated reference method.

The tested ultrafiltration units were:

a) Centrifree Micropartition System, Amicon Division, W. R. Grace & Co., Beverly (U. S. A.).

b) Centrisart-I, Sartorius, Göttingen (Germany).

c) Ultracent, Bio-Rad Laboratories, Richmond (U. S. A.).

d) Ultrafree-MC, Millipore Product Division, Bedford (U. S. A.).

Methods

Ultrafiltration

The Centrifree Micropartition System needs a fixed-angle rotor (JA-21 Beckman, 20 min at 1000 *g*) to provide an ultrafiltrate. The Ultrafree system was used in an Eppendorf (5414) centrifuge (60 min at 10 000 *g*) with a fixed-angle rotor.

The other two systems, Ultracent and Centrisart-I can produce an ultrafiltrate in a centrifuge with a swing-head rotor (40 min at 2900 *g*).

All four units carried a semipermeable membrane (cut-off $M_r \approx 10\,000$).

Because of the volume displacement effect of the proteins, we used a protein-dependent factor to adjust for this change in volume according to *Weast* (14).

Sample preparation

A serum sample (100–300 µl) was centrifuged for 20–40 minutes and 10 µl ultrafiltrate was directly injected into the HPLC system.

Direct injection of 5 µl of a serum sample onto the same system needs no preparation, except for turbid samples, which can be clarified with a Seraclear® filter (Bayer-Technicon, Tarrytown, U. S. A.). Sera were diluted with a Microlab M dilutor (Hamilton, Switzerland); 1000 µl serum were diluted with 1000, 2000, 3000 or 4000 µl NaCl, 154 mmol/l.

Total protein determination

We used the Technicon Chem-1 system for the total protein estimations (Biuret procedure).

High performance liquid chromatography (HPLC)

The HPLC system described in l. c. (5) was used. We used the same weak cation-exchange column, with two appropriate modifications: the pore size was changed from 7 to 11 nm, and the column dimensions were 100 × 4.6 mm I. D. and 50 × 4.6 mm I. D.

Flow rates were 1.0 ml/min for the 150 mm column, and 1.5 ml/min for the 100 mm and the 50 mm columns.

Statistical analysis

Results were compared by regression analyses according to *Passing & Bablok* (15). Significance of differences between medians of series of assay determinations was calculated with the *Wilcoxon* test for paired results; $p < 0.01$ was taken as significant.

Results

To evaluate the shorter columns we used a serum bank of 36 non-haemolytic, non-icteric and non-lipaemic sera with creatinine concentrations ranging from 40 to 1250 µmol/l; these samples were also analysed with our original method.

The use of a slightly larger pore size for the stationary phase improved the peak shape considerably. Tailing peaks (fig. 1) were abolished, thereby improving the resolution of the creatinine peak. In combination with the increased flow-rate of 1.5 ml/min, the retention-time of creatinine decreased from 5.6 min to 4.2 min on the 100 mm and to 2.8 min on the 50 mm column. The 50 mm column was therefore chosen, because only with this column were we able to increase the total number of analyses from 6 to 12 per hour.

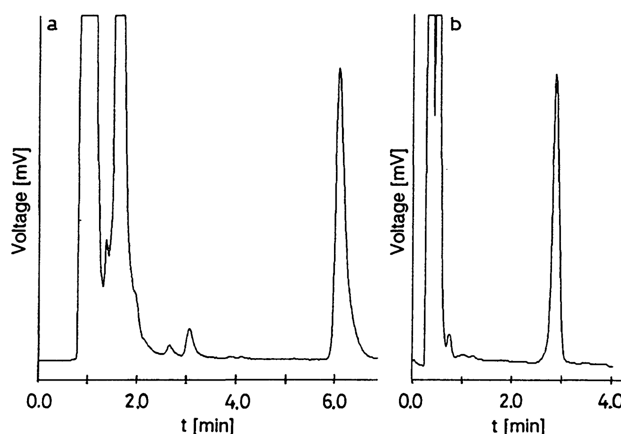

Fig. 1. The same ultrafiltrate analysed on the 150 mm colum (a), and on the 50 mm column (b), attenuation is 32 (2^5).

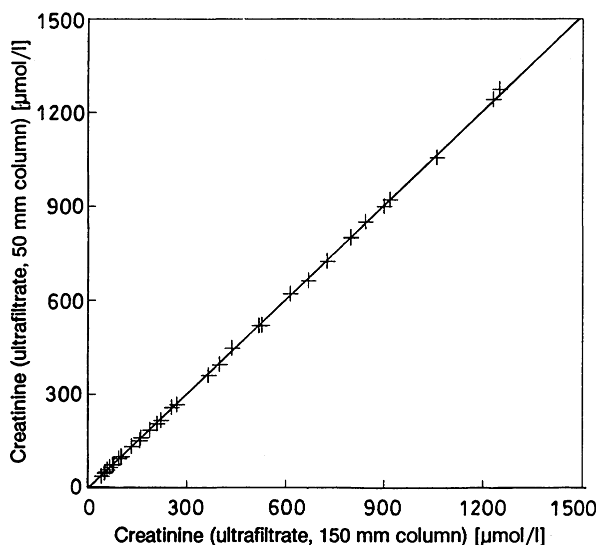

Fig. 2. The creatinine concentrations of 36 ultrafiltrates analysed on the 150 mm column (x-axis) and on the 50 mm column (y-axis), showing a perfect correlation.
$Y = 1.006x - 3.648$; $r = 0.99992$

Tab. 1. Method comparison (y = ax + b) between the Centrifree MPS unit (x) and three other ultrafiltration units (y).

Filtration Unit	a	b	n	r	Median x (μmol/l)	Median y (μmol/l)
Ultracent[a]	0.991 (0.987–0.994)	−0.017 (−0.486–0.941)	36	0.99996	237.4	235.0
Centrisart[b]	0.994 (0.988–0.998)	+0.171 (−0.762–1.103)	36	0.99987	237.4	237.4
Ultrafree[c]	1.177 (1.150–1.210)	−1.075 (−5.178–3.751)	36	0.99631	237.4	270.1
Ultrafree[d]	1.002 (0.953–1.024)	−0.956 (−3.154–5.326)	10	0.99967	90.8	87.3

[a] Ultracent significantly different from Centrifree ($p < 0.01$).
[b] Centrisart not significantly different from Centrifree.
[d] Ultrafree used in a cold room (4 °C), not significantly different from Centrifree.
[a–d] *Wilcoxon* signed ranks test.
Note: data between brackets are the 95% confidence limits.

The results of the ultrafiltrates analysed on the 150 mm and the 50 mm column are plotted in figure 2.

The correlation study showed an excellent regression line ($y_{50\,mm} = 1.006x_{150\,mm} - 3.648$), and 95% confidence interval ($r = 0.99992$), with 1.001 – 1.010 for the slope and −4.855 – −2.013 for the intercept. Median x = 237.4 μmol/l, median y = 236.2 μmol/l.

In a preliminary study we applied the Centrifree system, which suffers from the one drawback that it requires a fixed angle rotor centrifuge. We were therefore also interested in equivalent systems which only need a laboratory centrifuge with a swing rotor.

We used the 50 mm column to analyse the ultrafiltrates of the same 36 sera, and compared the results with those from the three other systems.

The Ultracent- and the Centrisart units gave nearly identical creatinine results, whereas the slope of the results from the Ultrafree unit showed a positive bias; we believe that this difference was due to the extremely long centrifugation time (60–80 min) at room temperature. When we repeated the experiment, with a subset of 10 serum samples in a cold room (4 °C, 60 min), the results from the Ultrafree unit were identical to those from the Centrifree unit.

Statistics are summarized in table 1.

The differences between the tested ultrafiltration units, when properly used, are negligibly small, but on basis of the *Wilcoxon* signed ranks test only the Centrisart unit was not significantly different from the Centrifree filter.

The remaining question was how to study the validation of the ultrafiltration step irrespective of the column used. Ultrafiltration of serum is used to protect the analytical column, and it results in a slight rise of the creatinine concentration, due to the protein displacement effect. To compensate for this effect we used a protein (dependent) factor. However, to measure the true creatinine concentration in serum, one has to inject the pure serum and consider this to be the golden standard. The correlation study of the 100 mm and the 150 mm column showed again an excellent regression line: $y_{100\,mm} = 1.005x_{150\,mm} - 3.558$, $r = 0.99944$ (95% confidence interval), with 0.996 – 1.016 for the slope and −4.397 – −0.742 for the intercept. Median x = 237.4 μmol/l, median y = 238.7 μmol/l.

Therefore we used our experimental 100 mm column to compare a series of ultrafiltrates and pure sera, and we investigated whether the application of pure serum could be used to validate the protein correction factor.

The same 36 sera, together with 3 control sera, were analysed before and after ultrafiltration.

The Centrifree units were used to produce the ultrafiltrates.

The only drawback was the rapid deterioration of the column, which became very noticeable after about 70 injections. Two typical chromatograms are shown in figure 3. Nevertheless, we were able to use the column again after a rigorous clean-up (24 hours of dilute phosphoric acid pH 3 and 8 hours 100% methanol).

The creatinine results correlate almost perfectly between pure serum and the ultrafiltrates, with a bias on the slope of only 1.050.

Correction with a protein-dependent factor (tab. 2), or even with a fixed protein factor (0.9465) removed all significant differences between the pure sera and the ultrafiltrates, confirming that the difference between ultrafiltrates and the native sera is mainly due to the protein displacement effect.

Because of the minor difference between the two calculation techniques we think that for routine analyses the use of a fixed protein factor is a valid option. The regression results are shown in table 3.

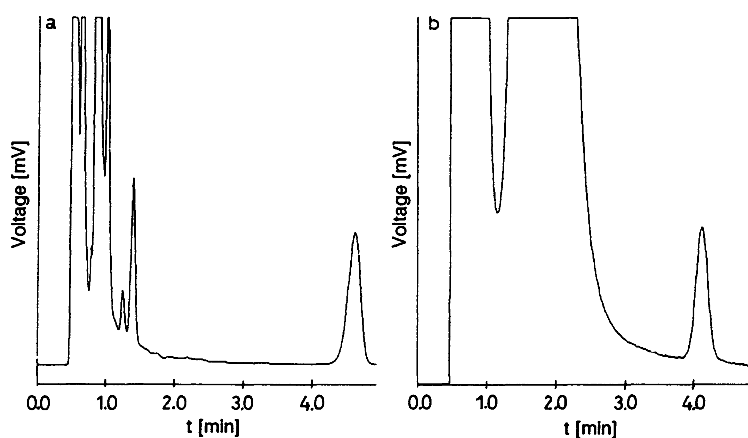

Fig. 3. A chromatogram of pure serum on a new column (a), and a chromatogram of pure serum after ± 70 injections (b), analysed on the 100 mm column; attenuation is 8 (2^3), injection volume 5 μl. The deterioration of the column can be seen in the void peak, and in a slight decrease in retention time.

Tab. 2. The correction of the creatinine concentration, on the basis of the total protein content of 36 sera, as described by *Weast* (14).

Sample	Protein (g/l)	Factor[1]	Filtrate (μmol/l)	Filtrate[2] (μmol/l)	Direct[3] (μmol/l)
1	58	0.9566	43.7	41.8	39.1
2	52	0.9606	53.7	51.0	50.2
3	74	0.9444	70.8	66.9	66.7
4	69	0.9485	83.3	79.0	77.8
5	68	0.9485	99.6	94.4	95.8
6	67	0.9495	139.6	132.5	130.1
7	80	0.9404	171.0	160.8	162.0
8	46	0.9657	194.6	188.0	185.4
9	63	0.9525	231.8	220.8	222.1
10	68	0.9485	284.9	270.2	270.3
11	53	0.9606	380.0	365.0	359.7
12	63	0.9525	418.3	398.5	400.2
13	55	0.9586	541.2	518.8	520.5
14	72	0.9465	647.7	613.0	615.1
15	71	0.9465	766.5	725.4	729.4
16	71	0.9465	842.5	797.4	804.6
17	65	0.9505	946.9	900.0	892.1
18	70	0.9465	1119.4	1059.4	1054.8
19	55	0.9586	1303.1	1249.1	1223.3
20	66	0.9505	79.2	75.3	73.5
21	63	0.9525	63.0	60.0	57.4
22	43	0.9677	53.0	51.3	50.4
23	71	0.9465	55.7	52.7	49.2
24	73	0.9444	108.6	102.6	98.2
25	81	0.9394	166.8	156.7	154.5
26	68	0.9485	105.6	100.1	98.9
27	73	0.9444	81.2	76.7	73.4
28	59	0.9545	220.1	210.1	206.8
29	70	0.9470	268.4	254.2	251.6
30	66	0.9505	459.7	436.9	432.7
31	70	0.9470	842.0	796.9	772.2
32	60	0.9545	882.7	842.6	856.9
33	55	0.9586	1283.6	1230.4	1219.4
34	47	0.9646	693.3	668.8	671.6
35	63	0.9525	964.0	918.2	929.6
36	67	0.9495	555.4	527.3	525.2

[1] Factor as determined on basis of the protein content.
[2] Filtrate after correction with a protein-dependent factor.
[3] Injection of 5 μl serum direct onto the HPLC column.

Tab. 3. Method comparison (y = ax + b) of 36 sera analysed as pure serum (x) and as ultrafiltrate (y).

	a	b	n	r	Median x (μmol/l)	Median y (μmol/l)
Ultrafiltrate[a]	1.050 (0.178 – 3.119)	1.856 (1.043 – 1.057)	36	0.99979	236.9	250.1
Ultrafiltrate[b]	0.993 (0.986 – 1.000)	1.755 (0.168 – 2.949)	36	0.99979	236.9	236.5
Ultrafiltrate[c]	0.998 (0.991 – 1.006)	2.201 (0.643 – 3.315)	36	0.99988	236.9	237.4

[a] Ultrafiltrate not corrected, y significantly different from x ($p < 0.01$).
[b] Ultrafiltrates corrected with a fixed factor of 0.9456, y not significantly different from x.
[c] Ultrafiltrates corrected with a protein-dependent factor, y not significantly different from x.
[a–c] *Wilcoxon* signed ranks test.
Note: data between brackets are the 95% confidence limits.

Tab. 4. Accuracy of direct injection compared with that of the reference method and injection after ultrafiltration.

Pool	Reference value[a] (μmol/l)	Mean[b] (μmol/l)	SD (μmol/l)	CV (%)
Pure sera				
A	155	152.9	2.4	1.5
B	221	214.5	1.7	0.8
C	593	585.9	7.9	1.4
Ultrafiltrate				
A	155	152.5	1.3	0.9
B	221	213.5	2.2	1.0
C	593	588.4	3.9	0.7

[a] Values determined by the reference method used by the 'Deutsche Gesellschaft für Klinische Chemie'.
[b] Mean values are calculated from n = 5.

The accuracy of the modified procedure was investigated with 3 reference sera, and the results are summarized in table 4.

As the 100 mm column performed so well, we wondered if the costs of an analytical column could counterbalance the costs of the ultrafiltration units, if we used diluted sera to delay column deterioration.

Five dilutions (1 ml + 0 ml, 1 ml + ..., 1 ml + 4 ml) were made of 5 normal serum pools. The resulting creatinine concentrations ranged from 79 to 456 μmol/l.

We checked the precision of the dilutor by diluting a serum sample (1 ml + 2 ml) 9 times and found a mean of 127.5 μmol/l, a SD of 0.54 μmol/l and a CV of 0.4%.

These results are within the normal within-run precision of this method (Kontrollogen L mean = 121 μmol/l, SD = 1.7 μmol/l, CV 1.4%).

Although the column performed for slightly longer with a reasonable selectivity (99 samples instead of 72), the experiments were not convincing. After 309 injections the column was unfortunately destroyed.

Discussion

The use of a 50 mm column in combination with the increased flow of 1.5 ml/min made it possible to cut down the overall run time for one creatinine determination from approximately 9 min to 4.5 min. This means that in an "emergency", the method is now more suited for problematic routine samples.

Using the short column, the time for incubation, calibration with 3 standards and the measurement of the sample and a control sample is now less than 1 hour.

The choice of the ultrafiltration unit is arbitrary. On basis of the *Wilcoxon* test, we found no difference between the Centrifree and the Centrisart filter. We therefore prefer the Centrisart unit because it produces an ultrafiltrate in a simple laboratory centrifuge.

The price of an ultrafiltration unit is high (± Dfl 5.00 per unit). An HPLC column is also expensive. The break-even point of the HPLC column is about 400 injections, but we did not reach that point. After approximately 300 injections and 3 clean-ups, the peak shape was such that we decided to replace the column.

Nevertheless the use of direct serum injection is possible, and it is a very simple and reliable way to evaluate reference and control sera.

In conclusion, for the determination of creatinine, HPLC with a modified weak cation exchanger and a column length of 50 mm or even less, is faster than the modification we described earlier, and peak-shape is improved considerably. Application of a fixed protein factor means that it is also a simple and sufficiently accurate back-up system for patient sera. Ultrafiltration is necessary to obtain a clean HPLC sample. When ultrafiltered samples are used, the column has an almost infinite lifetime (our first 150 mm column is still in use after ± 4000 injections.

Direct serum injection circumvents the use of a protein correction factor and is in our view an elegant way to upgrade our method to a reference method for the evaluation of reference material.

Acknowledgement

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L. Zwang
Academic Hospital Rotterdam-Dijkzigt
Department of Clinical Chemistry
Dr. Molewaterplein 40
NL-3015 GD Rotterdam
The Netherlands