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Journal of Chromatography B, 700 (1997) 261–268

JOURNAL OF
CHROMATOGRAPHY B

Short communication

Measurement of oxalate in human plasma ultrafiltrate by ion chromatography

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Received 7 February 1997; received in revised form 26 May 1997; accepted 26 May 1997

Abstract

An improved ion chromatographic method for the measurement of oxalate in human plasma ultrafiltrate is described. Ultrafiltration was carried out using an appropriate device and procedure. Centrifugation of 0.5 ml heparin plasma at 4°C for 50 min yielded water-clear ultrafiltrate in amounts allowing replicate measurements of oxalate. The specificity of the method was confirmed. The recovery of oxalate added to plasma was approximately 80%, whereas dilution of plasma, and of an oxalate-containing salt solution, resulted in falsely high values; the mechanism(s) underlying this phenomenon are insufficiently understood at present. The intra-assay precision of the method was assessed and from ten replicates of a pool plasma, the inter-assay precision from ten measurements of the same plasma on different days; the observed ranges of oxalate were 1.32–1.56 (mean 1.42) and 1.42–1.64 (mean 1.53) μmol/l, respectively. In plasma ultrafiltrate of a limited number of healthy volunteers the range of oxalate was 1.18–2.50 μmol/l, thus permitting renal oxalate handling to be studied. © 1997 Elsevier Science B.V.

Keywords: Oxalate

1. Introduction

Oxalate plays a predominant role in the formation of calcium-containing intrarenal and urinary tract concretions of humans and a number of mammalian species. In humans, calcium oxalate is the major constituent of calcium uroliths. Therefore, for a better understanding of oxalate handling by the kidneys, reliable measurement of plasma oxalate concentration is indispensable. In order to establish a correct diagnosis, mild hyperoxalemia must be reliably distinguishable from normo-oxalemia and early oxalosis (for details see Ref. [1]). However, for a number of reasons, the analytical methods applicable to plasma oxalate that are presently available do not satisfy this requirement and the obtained oxalate values are often misleading.

Enzymatic methods, although specific, require special instrumentation and technology, and are thus expensive. Furthermore, being susceptible to such interference factors as inhibitory active metal cations [2], and others [3], they are not quantitative. In the past, due to the instability of plasma oxalate during sample processing the resulting values were erroneous [4,5]. Finally, in a number of earlier publications dealing with oxalate analysis using chromatography, the specificity of the method was not adequately addressed.

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PII S0378-4347(97)00310-1

At present, suppressed anion-chromatography (IC) is the preferred method. However, in contrast to the measurement of urinary oxalate, plasma oxalate can be measured only after prior removal of accompanying proteins; among the preferred procedures are sample acidification [6] or extraction of oxalate [7,8] and ultrafiltration [9]. During the past few years we have gained experience with a variety of procedures for processing human plasma. Using appropriately prepared samples, we were finally able to obtain a reliable measurement of oxalate within the assumed physiological range (<1–3 μmol/l). We report on the details of this technically simple and specific method. This information is complementary to that described in our previous communications [8,9].

2. Experimental

2.1. Instrumentation

The ion chromatography system consisted of the following: Jasco 880 PU Intelligent pump (Jasco, Groß-Umstadt, Germany), the Rheodyne sample injection valve with a 10 μl sample loop (Bischoff, Leonberg, Germany), the pre-column IonPac AG4A-SC (50×4 mm I.D.), the analytical column IonPac AS4A-SC, 10 μm particle size (250×4 mm I.D.), the anion micro-membrane suppressor, and the conductivity detector (from ion chromatography system Dionex 2000 I). Except for the first two, all other items were from Dionex (Idstein, Germany). The Apex Computer Integration system was from ESWE (Sinsheim, Germany), the 10 kDa microconcentrator (Microcon-10) from Amicon (Witten, Germany), and the table centrifuge (type 5402) from Eppendorf Netheler-Hinz (Hamburg, Germany).

2.2. Chemicals

Only substances of the highest degree of purity available were used: oxalic acid, sodium oxalate, sodium tetraborate decahydrate, concentrated sulfuric acid, sodium carbonate and sodium bicarbonate (from Fluka, Neu-Ulm, Germany), oxalate oxidase (EC 1.2.3.4; Sigma Chemie, Deisenhofen, Germany). Only deionized bidistilled water was used as a solvent.

2.3. Collection, storage, pretreatment of plasma and plasma ultrafiltration

Blood was drawn from a forearm vein into a heparinized syringe, transferred to a prechilled tube, and immediately centrifuged at 4°C, and 3000 *g* for 3 min. Aliquots (500 μl) of the plasma were placed in Eppendorf vials, snap-frozen in liquid nitrogen, and stored at −80°C. Plasma cannot be injected directly into the chromatographic system, because proteins block the active sites of the column and shorten its life. Also, deproteinization of plasma, either with concentrated acid, other salts or organic solvents, leads to a variable loss of oxalate due to oxalate coprecipitation. Interference by other anions during the separation of oxalate is a regular phenomenon. These problems made it necessary to measure oxalate in a less complex matrix than whole plasma. Therefore, plasma proteins were removed by ultrafiltration using a 10 kDa pore size filter, which reflects the sieve micro-architecture of renal glomeruli.

Prior to analysis, samples were thawed within 2–3 min, transferred to the microconcentrator and centrifuged at 4°C, and approx. 8000 *g* (10 000 rpm) for 50 min. Initial studies showed that [^{14}C]oxalic acid added to plasma was completely ultrafilterable at 4°C and up to 60 min centrifugation. The water-clear ultrafiltrate obtained was either immediately injected into the chromatograph, or snap-frozen in liquid nitrogen to prevent oxalate neoformation at room temperature.

2.4. Chromatographic conditions

Oxalate was separated from ultrafiltrate using the described chromatographic system, and sodium tetraborate (22 m*M*, pH 9.1) as eluent. The flow-rate was adjusted to 1.6 ml/min. Sulfuric acid 25 m*M* (3.5 ml of conc. H_2SO_4 in 5 l of water) was used for regeneration of the suppressor system, operated at a flow-rate of 8 ml/min. A background noise suppressor column (synonymous "pulse dampener") was inserted between the pump and the sample injection valve, to minimize the pulsatile noise from the pump, a potential source of error (see Section 3). Prior to the measurement of standards and samples, the system was equilibrated over a period of 2 h. On each working day the operational check of the

system included the confirmation of acceptable inter-assay variation of a control sample (oxalate standard and pool plasma), and of adequate sensitivity. Oxalate eluted after about 6 min. The detector output was 1 μs=full-scale=1 V. On completion of the analysis, the system was washed with the carbonate eluent, containing sodium bicarbonate (100 mg) and sodium carbonate (100 mg) in 150 ml of water to protect the separation columns from deterioration.

3. Results and comments

3.1. Specificity, sensitivity

Using the procedure described, oxalate elutes as a symmetrical peak after sulfate. Initially, addition of oxalate oxidase (pH optimum 2–4) to plasma or ultrafiltrate failed to abolish the oxalate peak. It is known that potential enzyme inhibitors, such as ascorbic acid and metal cations [2], are abundant in untreated and acidified plasma or ultrafiltrate, i.e. oxidase shows decreased activity even when the pH is in the optimal range. In contrast, when injecting plasma extract (for details of procedure see Ref. [8]) or plasma ultrafiltrate extract, collection of the oxalate-containing fraction from chromatography, addition of oxidase to the latter, re-extraction and re-chromatography, no peak emerged (Fig. 1). Thus the peak observed in untreated samples is oxalate. This finding also confirms that oxidase develops sufficient activity when it is added to oxalate freed from, but not when associated with, organic matrices such as plasma or ultrafiltrate.

Because with the tetraborate eluent baseline noise was higher than that observed with the carbonate eluent, the suppressor regenerant flow-rate was increased to 8–10 ml/min. Using the tetraborate eluent and a peak-to-noise ratio of 4, an oxalate concentration of 0.75 μmol could be reliably read, and this value was therefore taken to be the lower limit of detection.

3.2. Oxalate in ultrafiltrate, completeness of the model

Fig. 2 shows that the oxalate concentration in the ultrafiltrate obtained with centrifugation periods of less than 50 min was higher compared with periods of 50 and 60 min, at which the concentration reached a plateau. Obviously, with the onset of centrifugation the used filter alters the distribution of solutes and macromolecules in plasma water. With longer centrifugation, further decline of measured oxalate was less than 5%. Thus, 50 min centrifugation was adopted as the standard procedure. After this time the ratio of oxalate calculated and oxalate measured becomes unity, as is demonstrated by the data in Table 1: the ultrafiltration device in use yields values close to those predicted by the conceptual model [10]. Assuming that the volume of protein in human plasma is approximately 7% (w/w), an Ox-C/Ox-A ratio of somewhat more than 1.0 would result, i.e. oxalate in the 50 min ultrafiltrate of unknown samples is slightly underestimated relative to oxalate in plasma. For practical purposes, no corrections were made for protein volume, distribution of proteinaceous substances including peptides, other solutes and the Gibbs–Donnan dysequilibrium factor. However, the latter would need proper assessment when specifically studying oxalate handling by the kidney, for example.

3.3. Accuracy

3.3.1. Calibration

As a result of carbon dioxide evaporation during centrifugation, the pH of the plasma ultrafiltrate generally rises above 8.0. Therefore, for oxalate calibration sodium oxalate solutions were used (pH 7.5–8.5). It should be noted that calibration with oxalic acid solutions (pH 4–6) would result in a steeper slope of the calibration line, and thereby lead to falsely low oxalate concentration in unknown samples. This would be especially true with supra-physiological oxalate values.

3.3.2. Recovery of oxalate

These studies were carried out after the addition of microlitre amounts of dissolved oxalic acid (1, 2, 4 μmol/l) to plasma, reflecting oxalate concentrations still in the physiological range or slightly above; also the effect of dilution of plasma at a 1:1 ratio was evaluated. Table 2 shows that when the calibration was based on peak height – as is practiced in routine measurements – approximately 80% of the added

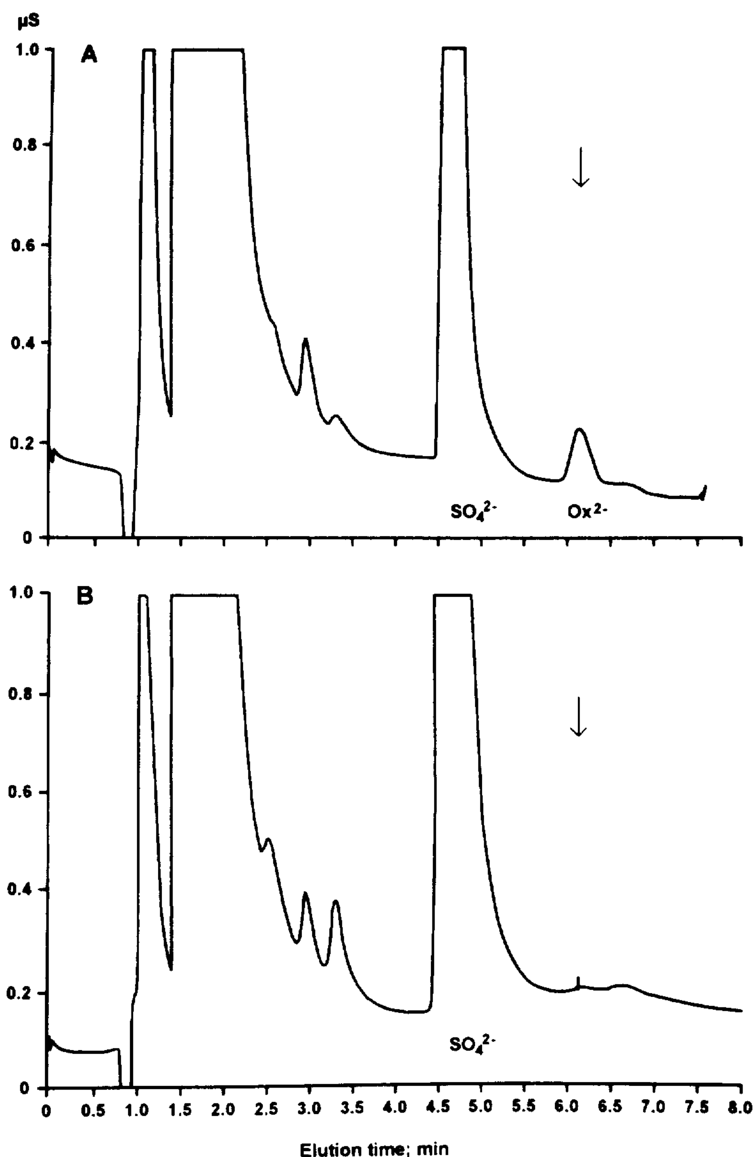

Fig. 1. IC of oxalate, and oxalate in plasma ultrafiltrate (validation ofthe ultrafiltration method). For details see text. (A,B): Elution profile of oxalate in diethyl ether extracts of human plasma, before (A) and after (B) addition of oxalate oxidase, and re-chromatography of the extract. ↓: position of oxalate.

oxalate concentration was constantly recovered. Conversely, when the calibration was based on peak area, oxalate recovery was 86% upon addition of 1 μmol/l, it was higher on addition of 2 μmol/l, and almost complete on addition of 4 μmol/l. In contrast to the findings with oxalate addition, a 1:1 dilution of each of ten different plasma samples resulted in higher measured vs. calculated oxalate. This was true for calibration in terms of peak height as well as in terms of peak area; the respective values are 157±(standard deviation) 18 and 129±34%.

Ascorbic acid, an important in vivo antioxidant present in plasma can lead to an unspecific increase in oxalate. With increasing aqueous sample dilution,

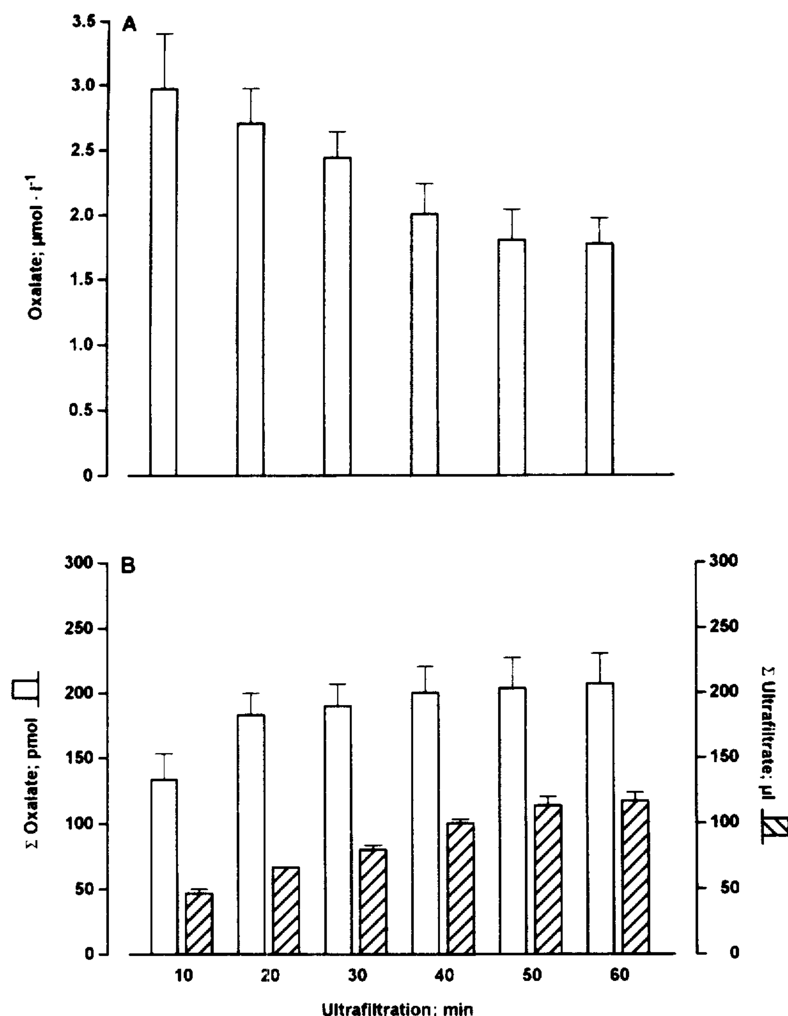

Fig. 2. (A) Oxalate concentration in ultrafiltrates of plasma from 6 individuals, obtained by centrifugation (10, 20, 30, 40, 50, 60 min) at 4°C. Columns indicate mean values and 1 standard deviation. (B) Cumulative ultrafiltrate volume (Σ; hatched columns) obtained from fractionated (10 min steps) centrifugation of plasma of each of the above-mentioned 6 individuals, and the cumulative oxalate mass in these ultrafiltrates (Σ; open columns). Note that the oxalate yield is almost constant between 50 and 60 min of centrifugation.

ascorbic acid restores the oxidative capacity of, for example, plasma tocopherols [11,12], a process that enhances the oxidative degradation of ascorbic acid to dehydroascorbic acid. Its transformation to diketogulonic acid and oxalate is a documented pathway [13]. Since the plasma concentrations of ascorbate and oxalate are respectively in the millimolar and micromolar range, a small excess of oxalate resulting from ascorbate degradation can explain at least a part of the disproportionately high oxalate recovery in diluted ultrafiltrate (Table 2). This could explain the high recovery of oxalate upon dilution, irrespective of the type of calibration.

We attempted to learn more about the factor(s) underlying the difference of percentage oxalate recovered on dilution, when peak height was compared with peak area calibration. Oxalic acid was added to water, or to a solution containing the anions chloride, phosphate, sulphate (0.1 *M* NaCl, 1.0 *M* NaH_2PO_4, 0.65 *M* Na_2SO_4, dissolved in water). With these ions the peak height of oxalate standard was reduced by 32% [oxalate in water (26 µmol/l)

Table 1
Evaluation of oxalate determination in plasma ultrafiltrate of 6 healthy males

Individuals No.	Ultrafiltration (min)	Ox-A[a] (μmol/l)	Σ Volume (μl)	Σ Ox (pmol)	Ox-C[b] (μmol/l)	Ox-CCC/Ox-A
1	50	1.63	137	227.3	1.66	1.018
2	50	1.50	101	147.7	1.46	0.975
3	50	2.10	131	227.8	1.74	0.828
4	50	1.18	133	162.7	1.22	1.037
5	50	2.49	148	393.1	2.66	1.067
6	50	1.94	136	227.7	1.67	0.863
Mean 1–6		1.81	131	231.0	1.74	0.964[c]
S.D.		0.47	16	87.1	0.49	0.097

[a] Analysis directly after the indicated duration of ultrafiltration.
[b] Calculated from the sum (Σ) of oxalate mass contained in the five 10 min fractions.
[c] A value of 1.04 is obtained after correction for the volume of mean plasma total protein (see Section 3).
For details see text.

compared with oxalate in salt solution (18 μmol/l)], contrasting with only 5% reduction of oxalate peak area (data not shown). With increasing dilution of the salt solution (1:1, 1:2, 1:3) the oxalate peak height showed approx. 12% higher values than those calculated from the dilution factors (63, 46, 37%), whereas the oxalate peak area showed values in proportion to the dilutions (50, 34, 26%). This difference in recovery percentages means that the oxalate peak shape varies in a largely constant manner and independent of the concentration of anions accompanying oxalate in a given sample. The underlying cause(s) are unknown at present.

3.3.3. *Precision (intra-, inter-assay variation)*

For this step calibration was based on peak height, since an almost constant percentage recovery, independent of the prevailing oxalate concentration, was demonstrated (see also Table 2). With 10 replicates of a serially measured pool plasma the oxalate range was 1.32–1.56 (mean 1.42) μmol/l; when, instead, these samples were measured based on peak area calibration, oxalate ranged from 1.35 to 1.92 μmol/l (not shown). The same pool plasma, when studied on subsequent days, was found to range from 1.42 to 1.64 (mean 1.53) μmol/l. Duplicate determination of oxalate in 10 unknown samples, each containing oxalate <2.2 μmol/l, showed a calculated [$S.D.=\sqrt{(\Sigma d^2)/2n}$] precision of 0.16 μmol/l, or an approx. 12% variation around the mean plasma oxalate value (1.32 μmol/l).

4. Plasma oxalate in healthy humans

In males (age 20–62 years; $n=10$) oxalate was 1.18–2.49 (mean 1.81) μmol/l; in females (age 23–

Table 2
Accuracy of oxalate measurement in plasma ultrafiltrate

Oxalate added (μmol/l)	n[a]	Oxalate (μmol/l)		Recovery (%)		Peak height/ peak area[b]
		Calculated	Measured	Peak height	Peak area	
0	10		1.42			0.85
1	10	2.42	1.90	78.7	85.7	0.83
2	10	3.42	2.76	80.7	94.5	0.79
4	10	5.42	4.39	80.9	98.5	0.78
Unknown samples	10[c]		0.85–2.15			0.85–1.03
Dilution 1+1	10	0.43–1.08	0.60–1.85	134–180	98–182	0.92–1.42

[a] Replicates of pool plasma.
[b] As concentration ratio.
[c] Duplicate measurement of plasma from 10 individuals.

55 years; $n=6$) the range was 1.31–2.50 (mean 1.73) μmol/l. These values are somewhat lower than those reported previously by us using a different ultrafiltration device, centrifugation at a higher temperature and a more alkaline HPLC eluent [9].

5. Discussion and outlook

Earlier work on plasma oxalate determination involved the use of [^{14}C]oxalic acid infusion into the systemic blood circulation; calculation of the oxalate by the renal clearance formula revealed values <2 μmol/l in normal humans [14,15]. Provided one accepts that the mean renal glomerular filtration rate of healthy normals is approx. 100 ml/min, and that urinary oxalate excretion in humans is mostly less than 300 nmol/min, the order of magnitude of plasma oxalate mentioned in those reports is consistent with net tubular oxalate secretion (ratio of the clearance of oxalate and glomerular filtration rate approx. 1.5). For decades, this was considered as the standard in analytical work on plasma oxalate that needed to be matched by subsequently developed less invasive methods.

Using the technology described in the present work this goal has now been achieved. The conceptual model of ultrafiltration was described earlier by Toribara et al. [10]. It predicts that the total amount of ultrafiltered oxalate – calculated as the sum of oxalate contained in multiple fractions obtained during a pre-determined centrifugation period – is identical to that measured after unfractionated centrifugation. Owing to differences in material and sieve properties, commercially available ultrafiltration devices often deviate substantially from this ideal model, especially with short centrifugation periods. Therefore, the minimum centrifugation time had to be determined. With the ultrafiltration technique used, oxalate was freely ultrafilterable, as evidenced by the data shown for 50 min centrifugation (Fig. 2). Accordingly, the previously observed oxalate binding [9] was apparent rather than real, and can be eliminated by using a filter membrane with physical properties that are incommensurable with (artificially generated) oxalate binding. Furthermore, the specificity of the present measuring method has been verified.

Interestingly, there are reports in the literature of similar values of oxalate, determined using IC or other methods. However, to the best of our knowledge in none of these reports did investigators demonstrate the complete sequence of events necessary for the maintenance of oxalate stability once blood had been withdrawn, or were details given on the specificity and reliability of the method. Furthermore, earlier attempts to determine "true oxalate" showed that potential sources of error leading to falsely high values were outweighed by others that regularly led to falsely low values; hence, the results obtained were only apparently correct. For example, plasma acidification, dilution, addition of oxalate oxidase in buffer, oxalate extraction with organic solvents, resins, or charcoal, all falsely increase oxalate, and this is also true when plasma is exposed to room temperature for more than 15 min during ultrafiltration [9], or repeatedly thawed. Also, the removal of ascorbic acid from plasma via degradation by ascorbic acid oxidase increases oxalate by 150–200%, due to the conversion of dehydroascorbic acid to diketogulonic acid and oxalate. The major source of oxalate loss is seen with inadequate manoeuvres for plasma deproteinization.

IC employing conductivity detection, an established technology, suffers from the limiting factor of high baseline noise. Acceptable reproducibility of values near the detection limit is thus reliable only when the pressure pulsation of the analytical pump and eluent is reduced, thereby "smoothing" the recording of the baseline. These disadvantages prevent automation of the sample injection system. At present, no superior non-invasive method can compete with IC. Since the quality criteria of the present method meet the requirements of analytical chemistry, we recommend it for use in biomedicine, including clinical studies of oxalate pathophysiology. The economic situation in terms of costs and benefits appears acceptable, since a throughput of approx. 40 samples per day seems to be feasible, provided there is batch ultrafiltration of unknown samples and subsequent storage of filtrates in liquid nitrogen until analysis. Also, for future work, a reduction in centrifugation time should be possible. For example, preliminary work with oblique fixation of the filter membrane inside the ultrafiltration device has yielded a somewhat higher ultrafiltrate volume, and an Ox-C/Ox-A ratio around unity after 20 min centrifu-

gation – data that are closer to the conceptual model [10].

Acknowledgments

Supported by the University of Erlangen Hospital Research Funds, and the W. Sander Foundation for the Advancement of Medical Sciences, Munich, Germany.

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