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Monitoring of chemotherapy-induced proteinuria using capillary zone electrophoresis

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Abstract

Capillary zone electrophoresis (CZE) was investigated for its suitability to monitor proteinuria occurring during nephrotoxic drug therapy. Urine samples of tumor patients receiving chemotherapy consisting of carboplatin, etoposide, and ifosfamide were concentrated and desalted in microconcentrators and analyzed in two different alkaline CZE buffer systems. Reduction of electroosmotic flow (EOF) by the addition of putrescine increased the number of resolved protein peaks. Both CZE methods were linear between 2.5 and 50 μg/ml, exhibited satisfactory precision (relative standard deviation <10%) and were suitable for monitor the time course of proteinuria after chemotherapy administration. In contrast to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), CZE detected interindividual differences in protein patterns. Whereas these differences hampered a direct quantification of proteins in urine, they may contain information on the type or extent of kidney damage.

Keywords: Proteinuria, chemotherapy-induced; Proteins

1. Introduction

A variety of drugs can cause renal damage due to functional and structural alterations [1]. Patients receiving those drugs have to be monitored very carefully in order to reduce the risk of nephrotoxicity. This is particularly important for cancer chemotherapy, e.g. when platinum complexes are used that can damage the tubular system of the kidney [2,3]. Moreover, nephrotoxicity is often dose-limiting in chemotherapy regimens [4]. A suitable method for the early detection of nephrotoxic effects is the measurement of proteins in urine [5]. In healthy individuals, urine contains only small amounts of proteins [6,7]. Following drug-induced glomerular damage, urine levels of proteins originating from plasma can be elevated considerably. Following tubular lesions, enzymes released from damaged cells can be detected in urine, indicating the type and extent of damage [8–10]. In order to prevent patients from severe kidney failure, elevations of protein and enzyme levels in urine should be detected as early as possible.

For this purpose, sensitive, selective, and fast analytical methods are required. Flat-bed electrophoresis on agarose (AGE) or polyacrylamide-based

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gels (SDS–PAGE) is commonly applied to monitor small amounts of protein in biological liquids [11]. During the last decade, however, capillary zone electrophoresis (CZE) [12] has developed from a novel technique to a reliable analytical tool that is steadily gaining higher impact in clinical laboratories [13]. The high efficiency of capillary electrophoresis in the separation of proteins in combination with short analysis times and the lack of staining procedures required favors the use of this technique in protein analysis [14].

In clinical laboratories, capillary electrophoresis has mostly been applied to fast routine analysis of human plasma samples [15–19]. Using a buffer system with an alkaline pH in short capillaries, ten different plasma fractions could be separated in less than 3 min. In a previous study, we reported the resolution of human transferrin isoforms in human plasma using bare capillaries [20]. In urine, capillary electrophoresis has been used for the characterization of myoglobinuria [21] and Bence-Jones proteinuria associated with patients suffering from multiple myeloma [22].

The major shortcoming often limiting the utility of capillary electrophoresis in bioanalysis is the insufficient detection limit (LOD). With conventional UV detection, LODs in the 10^{-6} *M* concentration range can be achieved. Sample preconcentration steps prior to the final separation run are required to extend the scope of possible applications. Another drawback of urine analysis by CZE is the presence of large concentrations of highly mobile low molecular ions, which considerably increases the required analysis times and reduces the sample throughput [23]. Both problems can be circumvented by the use of microconcentrators, involving a centrifugation step through a membrane. This method can separate proteins from ions and small molecules, thereby increasing the protein concentration in the samples prior to analysis.

The aim of this study was to establish a CZE-based semi-quantitative analytical method to monitor low concentrations of urinary proteins during treatment with nephrotoxic drugs. Human transferrin was selected as a model protein. The results were compared with complementary data obtained from conventional flat-bed SDS–PAGE analysis.

2. Experimental

2.1. Chemicals

Ammonium persulfate (APS), 1,4-butanediamine (putrescine), glycine, mesityl oxide, sodium dodecyl sulfate (SDS), sodium hydroxide, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), human transferrin, and tris(hydroxymethyl)aminomethane hydrochloride (Tris–HCl) were obtained from Sigma (Deisenhofen, Germany). Acetic acid, acrylamide, boric acid, bromophenol blue, methanol, and *N*,*N*-methylenebisacrylamide were purchased from Merck (Darmstadt, Germany). Coomassie Brilliant Blue R 250 was from Fluka (Buchs, Switzerland). Water for the preparation of all solutions was taken from a Milli-Q™ Plus water purification system (Millipore, Bedford, MA, USA). All buffers were filtered through 0.22 μm membrane filters (Sartorius, Göttingen, Germany) and were degassed prior to use in an ultrasonic bath.

2.2. Sample collection

Sequential urine samples were collected from ten male patients with germ cell cancer who were undergoing a high-dose chemotherapy cycle consisting of 3×500 mg/m^2 carboplatin, 4×600 mg/m^2 etoposide and 4×2.5 g/m^2 ifosfamide [24]. Blank urine samples were collected before the start of chemotherapy. The samples were stored at −70°C until required for CZE and SDS–PAGE analysis.

2.3. Sample preparation

All urine samples including spiked samples were centrifuged at 2200 *g* for 5 min in a Megafuge 1.0R centrifuge (Heraeus-Sepatech, Kansas City, MO, USA) in order to remove all non-soluble particles. Afterwards, samples were concentrated and desalted in Centricon 30 microconcentrators (Amicon, Houston, TX, USA) with a cut-off of 30 kDa. A 2-ml volume of urine was placed in a microconcentrator and centrifuged at 5000 *g* for 45 min in a Model

JA-218 centrifuge (Beckman Instruments, Palo Alto, CA, USA) equipped with a restricted-angle rotor, type JA 17 (Beckman Instruments). The supernatant was removed, and 2 ml of 10 m*M* borate buffer, pH 8.5, were added to the concentrated sample and the centrifugation was repeated. Afterwards, the volume of the concentrate was determined (about 50 μl) and the concentrate was stored at −70°C until analysis. All centrifugation steps were carried out at 15°C. In order to saturate residual unspecific binding sites on the membrane filter's surface, urine from a healthy volunteer, which was spiked with human albumin (100 μg/ml), was centrifuged through the membrane before the first sample was processed. After use, the microconcentrators were placed in an aqueous solution of 100 m*M* of sodium hydroxide for 5 min and subsequently rinsed with water. Using this procedure, the microconcentrators could be used several times.

2.4. Capillary zone electrophoresis

All CZE experiments were performed on a P/ACE 2100 system (Beckman Instruments). Fused-silica capillaries with an I.D. of 50 μm (Grom, Herrenberg, Germany) were cut to a length of 57 cm (length to the detector, 50 cm). For detection, UV absorbance was monitored on-line at 200 nm through a window that was burned into the outer polyimide coating of the capillary. CZE was carried out at a potential of 25 kV. Between runs, the capillaries were consecutively rinsed with 100 m*M* sodium hydroxide for 1 min and with separation buffer for 2 min. During analysis, the capillary was held at 25°C. The temperature of the samples in the auto-sampler was maintained at 15°C.

Two different buffer systems were used: System A consisted of 100 m*M* boric acid adjusted to pH 9.0 using sodium hydroxide. System B additionally contained 2 m*M* putrescine. Injections were performed by applying a pressure of 0.5 p.s.i. (1 p.s.i.= 6894,76 Pa) for 5 s, corresponding to an injection volume of approximately 12 nl. Mesityl oxide (0.1%, v/v), which served as a neutral marker of the electroosmotic flow (EOF), was injected for 1 s before injection of the sample solution.

2.5. Calibration and quality control

Calibration samples were prepared from human transferrin stock solutions (2.0 mg/ml) and diluted with freshly centrifuged urine from a healthy volunteer. Peak areas (PAs) of these samples were measured and corrected for the migration rate:

$$\mathrm{CPA} = \frac{PA \cdot L}{t_m} \quad (1)$$

where CPA is the corrected peak area, L is the length to the detector, and t_m is the migration time. Weighted linear regression ($w = 1/y^2$) of CPA versus concentration was performed in order to construct calibration graphs over a concentration range from 2.5 to 50.0 μg/ml. The limit of quantification (LOQ) was defined as the lowest concentration that could be measured with a relative standard deviation below 20%.

Biological quality control samples (BQC) were prepared by pooling urine samples from patients. Aliquots of BQC samples (2 ml) were stored at −70°C until analysis. Within-day precision was assessed by ten serial centrifugations of BQC samples that were subsequently analyzed using both buffer systems.

2.6. SDS–PAGE

Discontinuous SDS–PAGE analysis was performed according to Laemmli [25] in a PROTEAN 2 electrophoresis system (Bio-Rad, Richmond, CA, USA) using a gel size of 100×100×1.5 mm. The separation gel contained 7.5% (w/v) acrylamide and *N*,*N*-methylenebisacrylamide, 0.1% (w/v) SDS and 375 m*M* Tris–HCl, pH 6.8. The stacking gel contained 4% (w/v) acrylamide and *N*,*N*-methylenebisacrylamide, 0.1% (w/v) SDS and 125 m*M* Tris–HCl, pH 6.8. All parts of the gel were polymerized with 0.05% (w/v) TEMED and ammonium persulfate, respectively. The electrode buffer comprised 190 m*M* glycine, 25 m*M* Tris and 0.1% (w/v) SDS, pH 6.8.

Aliquots of sample (20 μl) were mixed with 40 μl of a solution containing 62.5 m*M* Tris–HCl, pH 6.8, 2% (w/v) SDS, 5% (w/v) mercaptoethanol, 10% (w/v) glycerol and 0.01% (w/v) bromophenol blue,

and immediately heated in a boiling water bath for 3 min. Subsequently, 120 μl of electrode buffer were added. A 20-μl volume of this solution was finally added to the gel and electrophoresed at 150 V for 50 min. After electrophoresis, the gels were stained with 0.1 (w/v) Coomassie Brilliant Blue R 250 in a methanol–acetic acid–water solution (40:10:50%, v/v) for 1 h and then bleached in a methanol–acetic acid–water solution (10:20:70%, v/v) for 1 h [26].

3. Results and discussion

3.1. Peak separation and identification

Based on previous experiments with the model protein human transferrin [20], two different buffer systems were selected. Buffer B differed from buffer A only by the addition of 2 m*M* putrescine, which is known to reduce the EOF, a strong plug-like flow originating from the zeta-potential at the inner wall of fused-silica capillaries [12]. In Fig. 1A–C electropherograms using buffer A, in Fig. 1D–F those using buffer B are presented.

Fig. 1A shows the electropherogram of concentrated blank urine of a healthy volunteer using separation buffer system A. A group of several small peaks, which were identified as albumin according to their migration times, was detected. With buffer system B, only very small peaks were visible in the same urine sample (Fig. 1D). The model protein human transferrin gave an additional homogeneous peak in buffer A (Fig. 1B). In buffer B, transferrin was resolved into four peaks, each representing a differently charged glycoform of the protein due to varying numbers of sialic acid residues (Fig. 1E). This is in good accordance with results obtained earlier in our laboratory [20].

Fig. 1C and F show two representative electropherograms of pooled urine samples from patients after nephrotoxic chemotherapy (BQC samples) with either buffer. With both buffers, a number of peaks could be resolved that apparently were not eliminated through the pores of the membrane. Since our electrophoretic conditions were optimized for protein analysis, it can be assumed that the peaks represent proteins. It is also evident that reduction of the EOF by the addition of putrescine to the buffer led to the separation of a larger number of peaks. The analysis time, however, increased by threefold (Fig. 1F). The albumin fraction generated a broad and strongly subdivided peak at a migration time interval between 20 and about 25 min (not shown). Because of this considerable extension of analysis time, we did not monitor this fraction.

Since, following glomerular lesions, mainly plasma proteins are found in urine, we analyzed a blank urine sample spiked with 1% (v/v) human plasma. Fig. 2A shows that the peak pattern of this sample is comparable to pooled urine from patients after chemotherapy (Fig. 1C). This suggests that patients´ urine contained elevated levels of albumin as well as other proteins assigned to other fractions that are normally found in human plasma.

3.2. Linearity

Using urine samples spiked with human transferrin, calibration graphs covering the concentration range from 2.5 to 50.0 μg/ml were constructed for both buffer systems using ten data points each. For buffer system B, the sum of corrected peak areas (CPA) of all transferrin glycoforms was used to establish the calibration graphs. A good correlation was achieved between corrected peak areas and transferrin concentrations using both electrolyte systems. A typical calibration graph obtained with buffer A is characterized by $y = 0.086x - 0.015$ ($r = 0.997$), with buffer B we obtained $y = 0.280x - 0.075$ ($r = 0.998$), where y represents the corrected peak area (arbitrary units) and x the analyte concentration (μg/ml). The LOQ was 2.5 μg/ml for both buffer systems.

3.3. Precision

Although good calibration graphs could be established, the direct quantitation of transferrin in urine samples of patients was not possible since distinct peaks could not clearly be assigned to transferrin. Fig. 2B shows that other proteins are present in urine samples and exhibit a mobility similar to transferrin. Furthermore, in plasma, transferrin is mainly present in its tetrasialoform, which was not available as a pure substance for calibration.

In order to assess the precision of both methods,

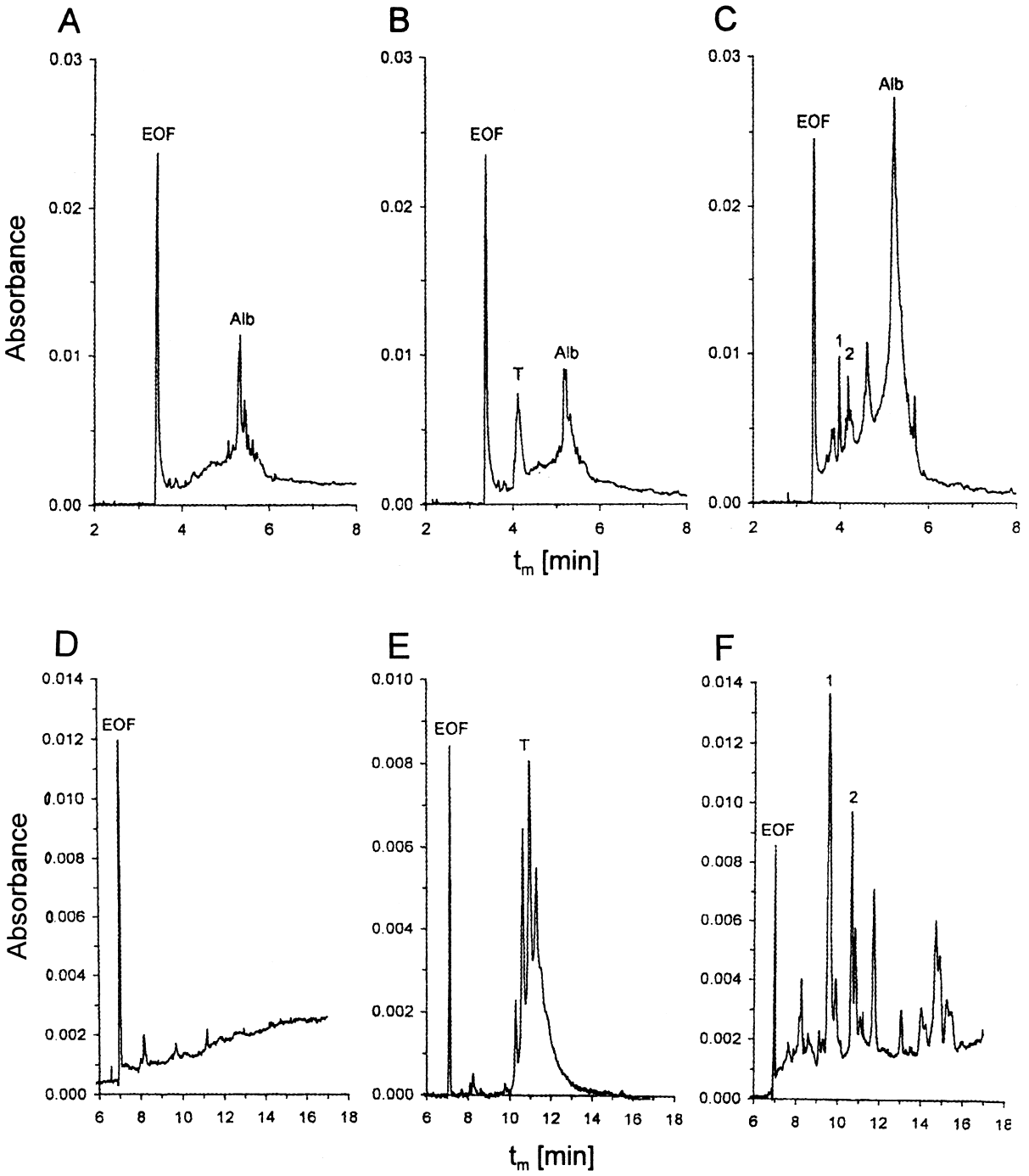

Fig. 1. (A–C) electropherograms obtained using separation buffer system A (100 m*M* borate buffer, pH 9.0), (D–F) using buffer system B (additionally contained 2 m*M* putrescine). (A, D) urine of a healthy volunteer, (B, E) the same urine spiked with 10 μg/ml of human transferrin and (C, F) a biological quality control sample prepared from pooled urine samples of patients undergoing nephrotoxic chemotherapy. Groups of peaks that were evaluated for precision are marked (1) and (2), (T) is transferrin and (Alb) is albumin. CZE conditions: uncoated fused-silica capillary, I.D. 50 μm, length 57 cm (50 cm to the detector), 25°C, hydrodynamic injection of 12 nl, 25 kV.

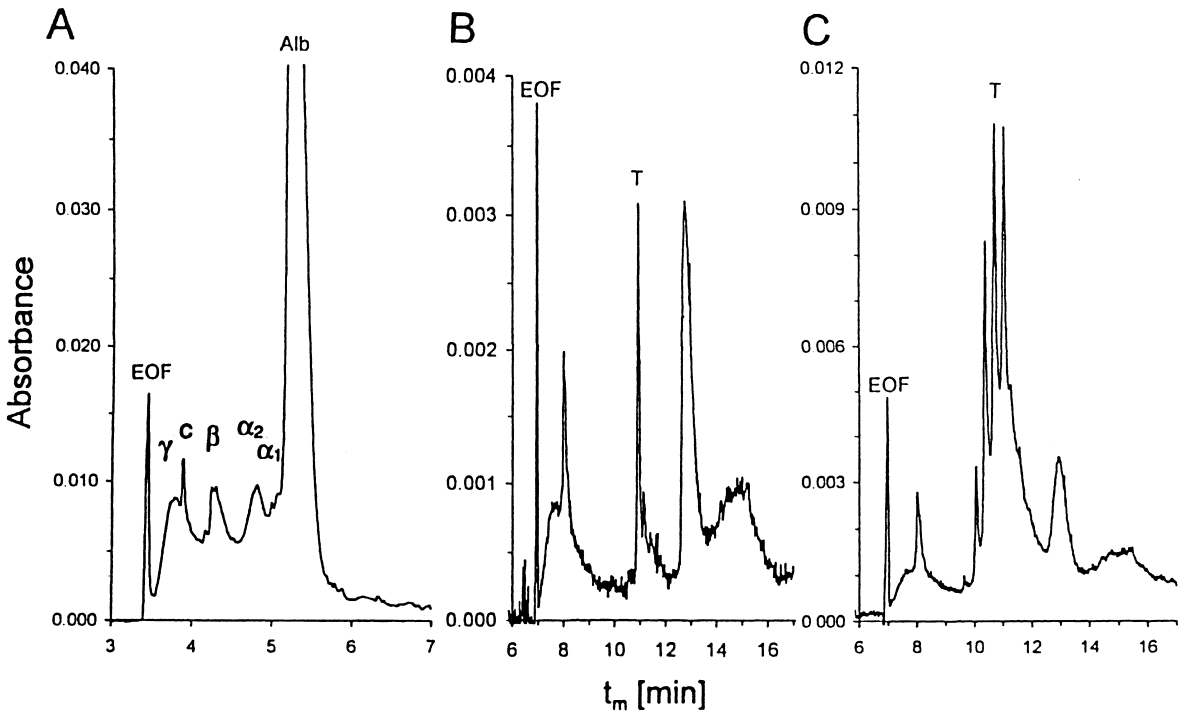

Fig. 2. Electropherograms showing the CZE analysis of a concentrated blank urine spiked with 1% (v/v) human plasma using (A) buffer system A and (B, C) buffer system B. In (A) the different plasma protein fractions are indicated ($\alpha_1 = \alpha_1$-globulins; $\alpha_2 = \alpha_2$-globulins; $\beta = \beta$-globulins; $\gamma = \gamma$-globulins; c = complement factors). In (C), 25 μg/ml of transferrin (T) was added. A different pattern of glycoforms than that shown in Fig. 1E was observed due to the content of tetrasialo-transferrin in plasma. For CZE conditions, see Fig. 1.

the CPA sum of characteristic peak groups was evaluated. Table 1 summarizes the within-day precision obtained by analyzing peak groups 1 and 2 in the electropherograms of the BQC sample (see Fig. 1C and F). The results indicate that the performance of the centrifugation membrane is not affected by repeated use.

3.4. Monitoring and quantification of proteinuria

Taking into account the advantages of either buffer system (buffer A, shorter analysis time; buffer B, better resolution), we decided to use them both for the analysis of individual urine samples from patients. Samples collected from ten patients undergoing potentially nephrotoxic chemotherapy were concentrated and analyzed by CZE and SDS–PAGE as complementary methods. Serum creatinine levels revealed nephrotoxicity grade 1 only for one patient (no.7). Nevertheless, an increase in urine protein levels was observed for all ten patients investigated. Electropherograms with buffer A of concentrated urine samples of four patients collected six days after the start of therapy are depicted in Fig. 3. Inter-individual comparison of all electropherograms revealed large differences in peak pattern among patients. This observation was also made with buffer B (not shown). It can be assumed that the different protein peak patterns observed in urine are related to individually different types of damage occurring to the kidney during chemotherapy. This could be due to the fact that the patients received two different

Table 1
Within-day precision of the two CZE assays investigated ($n = 10$)

CZE buffer	Peak group 1		Peak group 2	
	CPA[a]	RSD[a] (%)	CPA[a]	RSD[a] (%)
A	2.53	8.3	4.65	7.0
B	9.17	10.2	4.21	9.7

[a] CPA: corrected peak area; RSD: relative standard deviation.

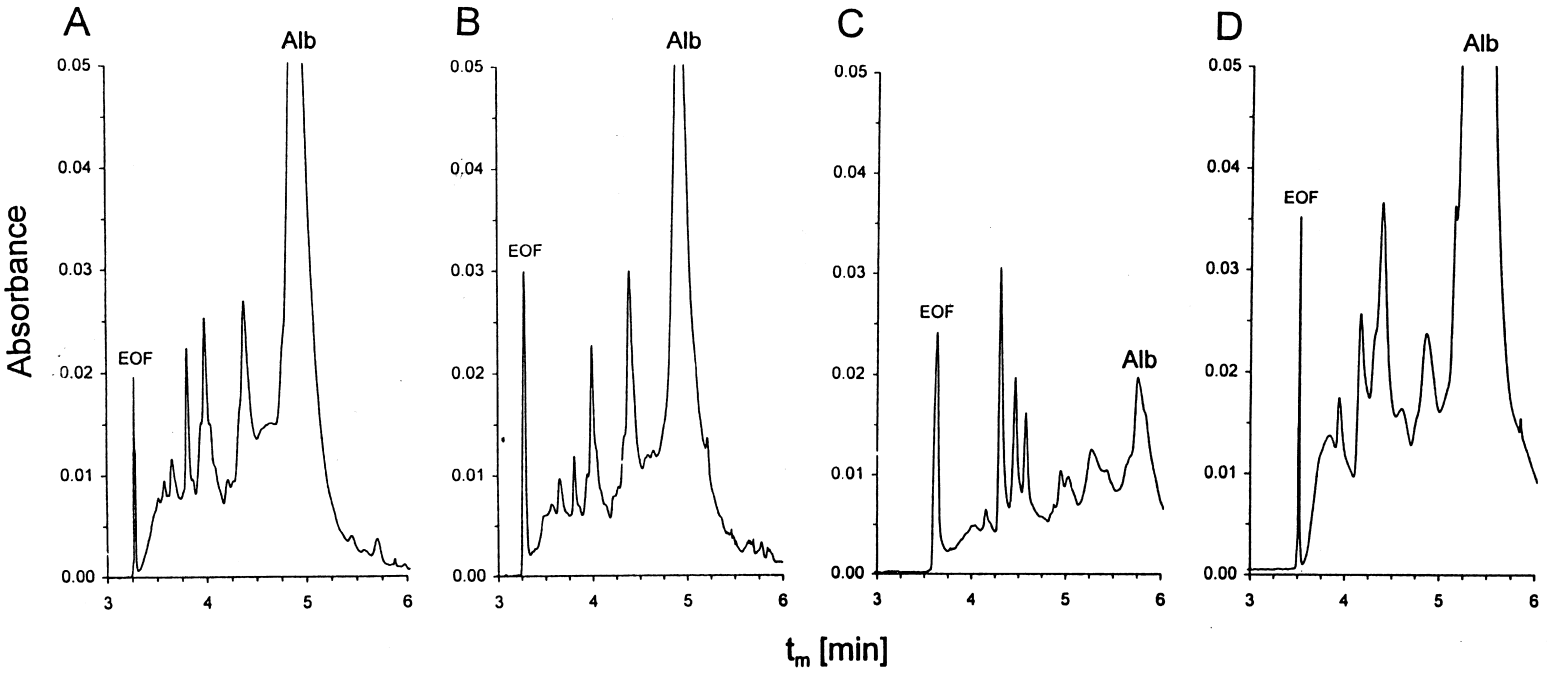

Fig. 3. CZE analysis of concentrated urine samples from four patients, collected six days after the start of chemotherapy using buffer A. For peak identification and CZE conditions, see Fig. 1

nephrotoxic drugs simultaneously, i.e. carboplatin and ifosfamide.

In an attempt to quantify the observed nephrotoxic effects during chemotherapy, we calculated the sum of all the corrected peak areas in the migration time interval related to the β-globulin fraction (ΣCPA), including transferrin. Based on our quantitative results with transferrin, it can be assumed that CPA correlates with protein concentration. Using the sum of CPA, we calculated the relative renal elimination rate (ER_β) of β-globulins using the following equation:

$$ER_\beta = \frac{V_U \cdot \Sigma CPA}{\Delta t} \quad (2)$$

where V_U is the volume of the urine sample, and Δt is the collection interval. Thus, $ER_ß$ is only a correlate of the *true* protein elimination rate.

The course of ER_β during the time of investigation for all investigated patients is shown in Fig. 4. It is evident that all patients developed proteinuria during chemotherapy. However, the kinetics of proteinuria differed considerably among patients. Most of the patients showed increased protein elimination rates on the second day after the start of chemotherapy, indicating that ER_β is an early marker of kidney damage. The patient exhibiting nephrotoxicity grade 1 showed the highest $ER_ß$ measured. The electropherograms of the samples of this patient, with either CZE method before, during and after chemotherapy and a corresponding SDS–PAGE gel are shown in Fig. 5. Electropherograms 5A to 5C were collected using buffer system A, whereas 5D to 5F were generated using buffer B. β marks the fraction used for calculation of ER_β.

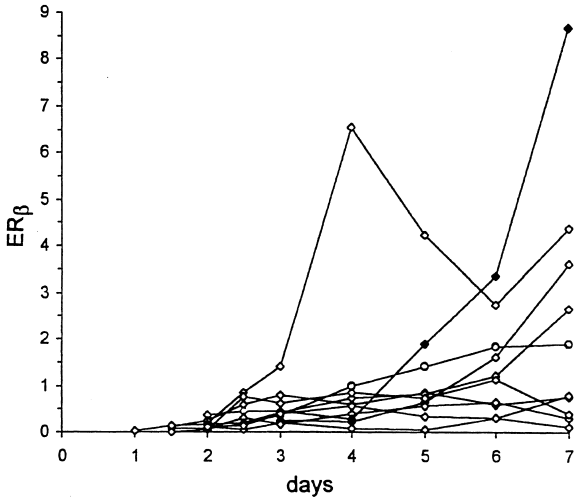

Fig. 4. Protein elimination rate correlate (ER_β) versus time elapsed after the start of chemotherapy. The patient exhibiting nephrotoxicity grade 1 is marked with solid symbols (electropherograms are shown in Fig. 5); the other patients are marked with open symbols.

The different separation mechanisms of CZE and SDS–PAGE did not allow for correlation between the identity of spots on the gels and certain peaks in the electropherograms. Compared to the respective electropherograms, the patterns found in the gels showed less divergence. SDS–PAGE was hence less suited to reveal individual differences among patients. However, the potential benefit of this information for the clinical situation is unclear and has to be assessed in future investigations. CZE reanalysis of fractions collected from analytical techniques with complementary separation mechanisms, like size-exclusion chromatography should provide additional information about unknown substances in urine. Alternatively, the application of mass spectrometric detection coupled to CZE, as recently reviewed [27], could supply more detailed information about the nature of the analytes.

4. Conclusions

CZE appears to be a powerful alternative to conventional flat-bed electrophoresis for monitoring urinary protein levels after nephrotoxic drug therapy. Both established CZE methods provided satisfactory linearity and precision. The time course of proteinuria could be characterized after administration of cancer chemotherapy. However, direct quantification of individual proteins was not possible due to interindividual differences in peak patterns. These differences suggest different mechanisms of nephrotoxicity and could not be resolved by SDS–PAGE analysis. Complementary data are required to permit a final evaluation of the potential of CZE for the monitoring of chemotherapy-induced proteinuria.

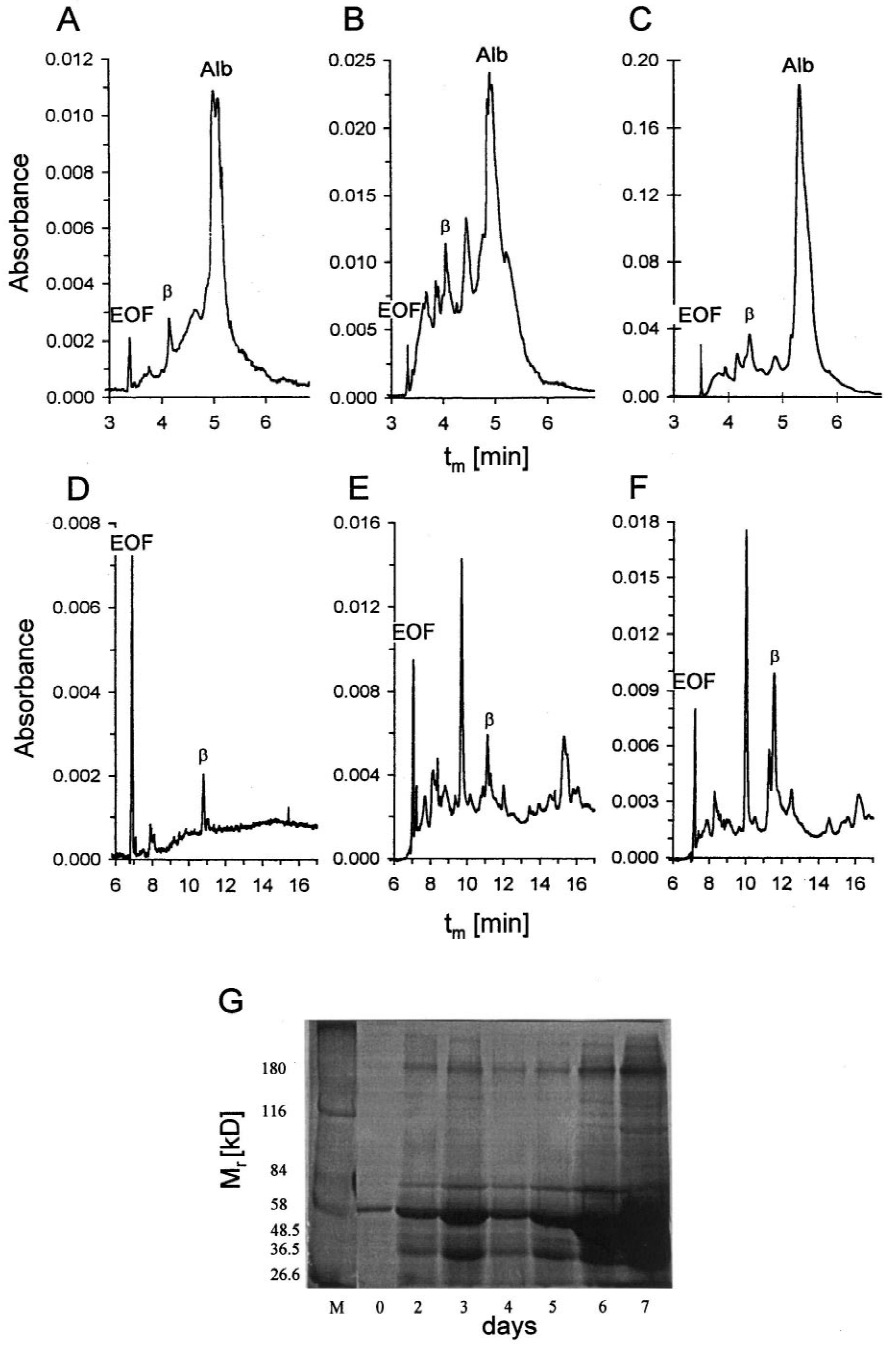

Fig. 5. (A–F): Selected electropherograms of concentrated urine samples from patient 7 (exhibiting nephrotoxicity grade 1). (A–C) were monitored using buffer system A, (D–F) originate from buffer system B. (A) and (D) are samples taken before, (B) and (E) three days, and (C) and (F) seven days after the start of chemotherapy. The group of peaks marked with (β) was used to estimate ER_g. (G) is the SDS–PAGE gel of urine samples from this patient over the investigated time interval of seven days. M indicates molecular mass marker proteins. For CZE conditions, see Fig. 1.

References

[1] M.A. Matson, P.A. Abraham, in: J.T. DiPiro, R.L. Talbert, P.E. Hayes, G.C. Yee, G.R. Matzke, L.M. Posey (Eds.), Pharmacotherapy, A Pathophysiologic Approach, 2nd ed, Appleton & Lange, Norwalk, CT, 1993, p. 701.

[2] G. Daugaard, U. Abildgaard, Cancer Chemother. Pharmacol. 25 (1989) 1.

[3] D.Th. Sleijfer, E.F. Smit, S. Meijer, N.H. Mulder, P.E. Postmus, Br. J. Cancer 60 (1989) 116.

[4] M.P. Goren, A.A. Forastiere, R.K. Wright, M.E. Horowitz, R.K. Dodge, B.A. Kamen, M.J. Viar, C.B. Pratt, Cancer Chemother. Pharmacol. 19 (1987) 57.

[5] D.C. Robinson, G.S. Jaresko, D.E. Baker, in: M.A. Koda-Kimble, L.Y. Young (Eds.), Applied therapeutics, the clinical use of drugs, 5th ed, Applied Therapeutics, Vancouver, WA, 1992, p. 27-1.

[6] T.S. Larson, Mayo Clin. Proc. 69 (1994) 1154.

[7] D. Maruhn, I. Fuchs, G. Mues, K.D. Bock, Clin. Chem. 22 (1976) 1567.

[8] R. Beetham, W.R. Cattell, Ann. Clin. Biochem. 30 (1993) 425.

[9] W.G. Guder, W. Hofmann, Clin. Nephrol. 38 (1992) S3.

[10] B.R. Jones, R.B. Bhalla, J. Mladek, R.N. Kaleya, R.J. Gralla, N.W. Alcock, M.K. Schwartz, C.W. Young, M.M. Reidenberg, Clin. Pharmacol. Ther 27 (1980) 557.

[11] L. Thomas (Ed.), Labor und Diagnose, Medizinische Verlagsgesellschaft, Marburg, 1992, p. 466.

[12] R. Kuhn, S. Hoffstetter-Kuhn, Capillary Electrophoresis: Principles and Practice, Springer Verlag, Berlin, New York, 1993.

[13] M.A. Jenkins, M.D. Guerin, J. Chromatogr. B 682 (1996) 23.

[14] J. Bergmann, U. Jaehde, W. Schunack, Electrophoresis 19 (1998) 305.

[15] F.T.A. Chen, J. Chromatogr. 559 (1991) 445.

[16] V.J. Dolnik, J. Chromatogr. A 709 (1995) 99.

[17] R. Clark, J.A. Katzmann, E. Wiegert, C. Namyst-Goldberg, L. Sanders, R.P. Oda, R.A. Kyle, J.P. Landers, J. Chromatogr. A 744 (1996) 205.

[18] E. Jellum, H. Dollekamp, C. Blessum, J. Chromatogr. B 683 (1996) 55.

[19] R. Lehmann, H. Liebich, G. Grübler, W. Voelter, Electrophoresis 16 (1995) 998.

[20] J. Bergmann, U. Jaehde, W. Schunack, Pharmazie 51 (1996) 644.

[21] Z.K. Shibabi, J. Chromatogr. B 669 (1995) 53.

[22] M.A. Jenkins, T.D. O'Leary, M.D. Guerin, J. Chromatogr. B 662 (1994) 198.

[23] F. Foret, E. Szöko, B.L. Karger, Electrophoresis 14 (1993) 417.

[24] W. Siegert, J. Beyer, I. Strohscheer, H. Baurmann, H. Oettle, J. Zingsem, R. Zimmermann, C. Bokemeyer, H.J. Schmoll, D. Huhn, J. Clin. Oncol 12 (1994) 1223.

[25] U.K. Laemmli, Nature 227 (1970) 681.

[26] D. Freifelder, Physical Biochemistry, Freeman, New York, 1982

[27] J. Cai, J. Henion, J. Chromatogr. A 703 (1995) 667.