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Determination of prednisolone in serum: method development using solid-phase extraction and micellar electrokinetic chromatography

S. Noé [a], J. Böhler [b], E. Keller [b], A.W. Frahm [a,*]

[a] *Chair of Pharmaceutical Chemistry, University of Freiburg, Hermann–Herder-Str. 9, D-79104 Freiburg im Breisgau, Germany*
[b] *Department of Nephrology, University of Freiburg, Hugstetter Str. 55, D-79106 Freiburg im Breisgau, Germany*

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1. Introduction

The immunosuppressive therapy following solid organ transplantation is being practised as a combined drug therapy regimen. Besides ciclosporin A, azathioprine and mycophenolate mofetil, the corticosteroids prednisone and prednisolone (Fig. 1) are used for immunosuppressive therapy. Beyond this, parenteral prednisolone is used for the treatment of acute rejection episodes [1–3]. On one hand, prednisone as a prodrug is converted in vivo to the active moiety prednisolone by endogenous enzymes. On the other side a small amount of the administered prednisolone will be converted to prednisone. It is wellknown, that the metabolism of corticosteroids highly depends on individual conditions [4–8]. The immunotherapy of acute rejection is done by a fixed drug regimen (500 mg prednisolonehydrogenesuccinate, e.g. Solu-Decortin H®) intravenous on first, third and fifth day of treatment). Following this regimen, there is a risk of subtherapeutic concentrations on one side and on the other side toxic side effects caused by overdosing.

Determination of serum levels of corticosteroids normally is measured by immunoassays. Many of these assays show significant cross reactions between endogenous steroids and administered steroidal drugs [9,10]. Chromatographic methods, especially high performance liquid chromatography (HPLC), were cited as reference methods [4–6,9–18]. A method for the determination of corticosteroids using micellar electrokinetic capillary chromatography (MECC) with the advantages of low-priced and less pollutant chemicals still has to be established. In principle, corticosteroids could be measured in serum without sample pretreatment (direct serum injection) [19], but due to low serum levels (microgram), preconcentration has to take place before determination of the steroids. Several buffer systems used for the

* Corresponding author. Tel.: +49 761 2036335; fax: +49 761 2036351; e-mail: awfrahm@sun2.ruf.uni-freiburg.de

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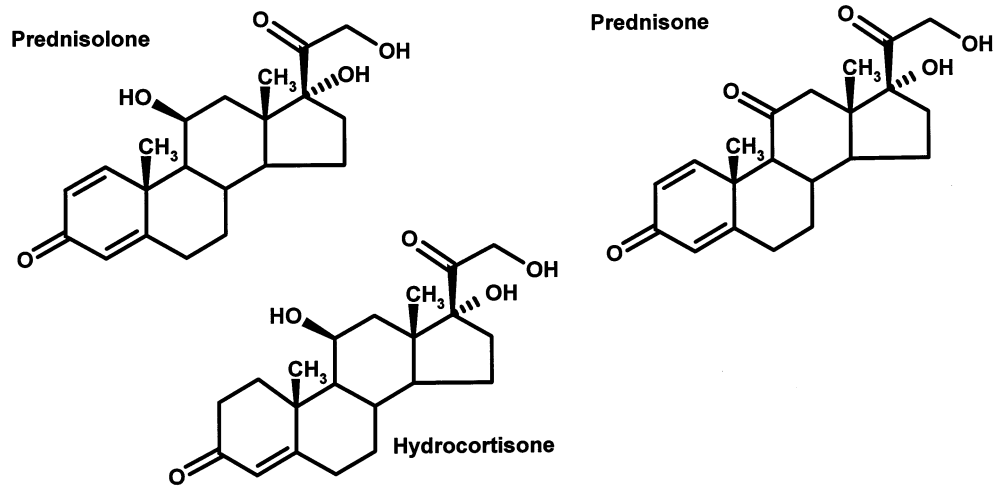

Fig. 1. Structures of the corticosteroids prednisolone and prednisone (drugs) and of the endogenous steroid hydrocortisone (cortisol).

determination of prednisolone with MECC have been developed [20–22]. Liquid–liquid extraction (LLE) and solid-phase extraction (SPE) were investigated as sample pretreatment. Up to date there were only three papers dealing with the separation, but none of them described the quantitative determination of steroids in biogenous specimen with MECC [23–25].

2. Experimental

2.1. Chemicals

Prednisone and prednisolone were gifts from E. Merck KG (Darmstadt, Germany) and hydrocortisone a gift from Schering AG (Berlin, Germany). Cortisone, dehydrocholic acid sodium salt (DHC) and taurocholic acid sodium salt hydrate (TC) were purchased from Fluka Biochemika (Neu-Ulm, Germany), sodium dodecyl sulfate (SDS) from BioRad (Richmond, USA). Methanol (Rotisolv®, Roth Karlsruhe, Germany) and acetonitrile (Janssen, Geel, Belgium) were of HPLC grade. Stock solutions of steroids were prepared in methanol to concentrations of 40, 80, 120, 160, 320 and 1000 μg ml^{-1} and stored at ca. 7°C.

2.2. Equipment

MECC was performed on Prince Crystal CE 310 capillary electrophoresis systems (ATI Unicam, Kassel, Germany) with autosampler and automatic outlet buffer changer. Separations took place in fused-silica capillaries (Laser 2000, Weßling, Germany) with i.d. 50 μm, total length 66 cm and effective length 49.5 cm. Detection was effected with Unicam 4225 UV-detectors (ATI Unicam, Kassel, Germany). Migration times and peak height acquisition were performed by ATI Unicam 4880 chromatography data handling system (version 2.04). Data evaluation and calculation were performed by MS-Excel 5.0 (WIN-OS/2) and MS-Excel 7.0 (WIN95).

2.3. Reagents

Buffer I: phosphate-tetraborate buffer (pH 8.0; 20 mM) with SDS (50 mM) and acetonitrile (16% v/v): Sodium dihydrogenphosphate hydrate ($NaH_2PO_4 \cdot H_2O$) (275 mg) was solved in 100.0 ml of water (solution 1). Sodium tetraborate decahydrate ($Na_2B_4O_7 \cdot 10\ H_2O$) (762 mg) was solved in 100.0 ml of water (solution 2). Solution 2 was added to solution 1 until the pH value reached 8.0 (solution 3). SDS (720 mg) was dissolved in a minimum of solution 3, 8.0 ml acetonitrile were

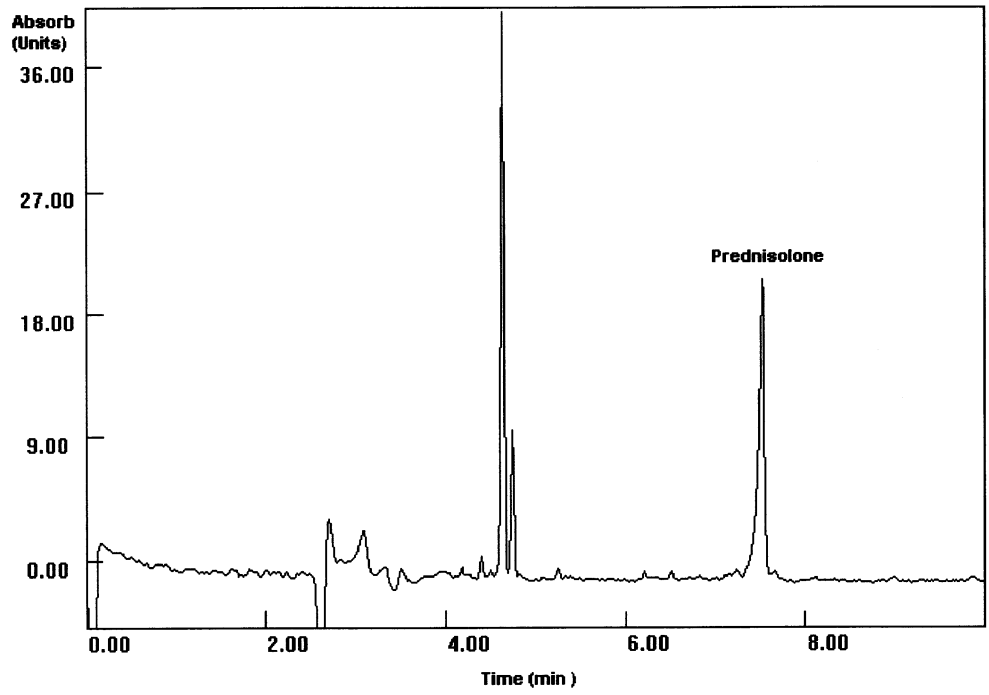

Fig. 2. Electropherogram of renal transplant's serum, treated with 500 mg Solu-Decortin H®, analysed in buffer I. Other conditions mentioned in text. Peak indicating prednisolone besides other unidentified substance peaks.

added and the resulting mixture was made up with another portion of solution 3 to 50.0 ml.

Buffer II: phosphate-tetraborate buffer (pH 9.0; 50 mM) with 50 mM of SDS, TC, DHC each: 690 mg $NaH_2PO_4 \cdot H_2O$ was solved in 100.0 ml of water (solution 4). A total of 1345 mg $Na_2B_4O_7 \cdot 10\ H_2O$ was solved in 100.0 ml of water (solution 5). Solution 5 was added to solution 4 until the pH value reached 9.0 (solution 6). SDS (720 mg), TC 1345 mg and DHC 1061 mg were made up to 50.0 ml with solution 6.

The conditioning of new capillaries was effected by rinsing the capillary with 0.1 M NaOH, water, 1 M HCl, water and then with the buffer (10 min each). Between run washes were performed by rinsing the capillary with 0.1 M NaOH (10 min), water (10 min) and then with the buffer (5 min).

2.4. Specimen

Patient blood samples were collected with 10 ml Monovetten® (Sarstedt, Rommelsdorf, Germany) and serum was prepared by centrifugation (5000 rpm; 30 min). The serum was stored at ca. −20°C.

2.5. Extraction

Isolute® SPE-columns with C_{18}-endcapped sorbents (ICT, Bad Homburg, Germany) were conditioned with 10 ml of methanol and 10 ml of water. Serum (4 × 1000.0 μl) was applied to the SPE-column and aspirated through the column using a Vac-Master® (ICT, Bad Homburg, Germany). The SPE-column was washed with 30 ml of water for removal of the proteins and other substances. The steroids were eluted with 3 ml of methanol and the elute was collected in a 5 ml vial. The solvent was removed under a gentle stream of nitrogen at 50°C in a home-made evaporator. The residue was reconstituted and carried over to a microvial (500 μl) with methanol (3 × 100 μl). The solvent was also evaporated under nitrogen. For the second time the vial was washed

with methanol (2 × 100 μl) and the wash solution was carried over into the same microvial. Methanol was removed under nitrogen and the residue was dissolved in 50.0 μl of methanol. This sample was mixed and sonicated and then put into the autosampler for analysis. The temperature of the autosampler was controlled at 20°C (±0.1°C). With this extraction procedure the steroids were concentrated by the factor 80. In some cases it was possible to concentrate the samples up to 200 times, but often the solubility of the substances restricted the sample volume reduction.

2.6. Analysis

The prepared sample was injected into the anode capillary inlet under pressure (20 millibar, 0.06 min). The autosampler was tempered at 20°C (±0.1°C) and the capillary oven at 25°C (±0.1°C). Automatic outlet buffer replenishment was performed with the butler. Buffer I has been used for the quantitative determination of prednisolone. Separations were performed under constant voltage of 30 kV with a current of ca. 65 μA during 10 min. Detection was effected at 254 nm. Calibration samples (40, 80, 120, 160 and 320 μg ml^{-1} prednisolone in methanol) were analysed within the same sample strip with the serum samples. These concentrations are equivalent to prednisolone concentrations gained after a 80-fold concentration of a serum containing 0.5, 1, 2, 3 or 4 μg ml^{-1} prednisolone.

Furthermore, separations have also been performed with buffer II. These separations took place under constant voltage of 15 kV (current ca. 70 μA) during 60 min. Injection, capillary and detection were done likewise as with buffer I.

3. Results and discussion

The development of the method for the determination of prednisolone in serum using MECC and solid-phase extraction can be assessed as follows.

3.1. Evaluation of the developed procedure of extraction

The recovery of prednisolone following the extraction procedure and the analysis was examined by spiking a serum specimen of one of the authors with known doses of prednisolone drug. The samples were pretreated and analysed as described in the experimental part. Peak heights were compared to peak heights of prednisolone in calibration samples. The mean recovery rate was calculated to 74% (RSD 6.6%; $n = 3$), which is similar to that published by Garg and Jusko [17] (76%), for the HPLC separation technique.

3.2. Comparison of the buffers

As published earlier [19,20], buffer I and buffer II differ in specificity and separation times. For quantitative determination of prednisolone in serum, buffer I has been used, even though the endogenous hydrocortisone could not be separated from exogenous prednisolone. However, this interference is not of importance for clinical practise, since patients, undergoing high-dose therapy with prednisolone, have very low serum levels of hydrocortisone (below the detection limit of 250 ng ml^{-1}).

Advantages of buffer I were the very short separation time (< 10 min) and the low costs of running buffer additives.

Fig. 2 shows a typical electropherogram of a SPE-pretreated serum of a patient treated with 500 mg Solu-Decortin H® (peak level). Following SPE, the sample was analysed in buffer I. Migration time of prednisolone was 7.43 min (RSD 1.2%; $n = 5$). Peak height of prednisolone was 18.72 units (RSD 11.5%; $n = 5$). Identity of the prednisolone peak was elucidated by spiking the sample with the reference compound. Furthermore, the migration time of the prednisolone peak was evaluated with buffer II. Fig. 3 shows a typical electropherogram of a pretreated serum, analysed in buffer II. Migration time of prednisolone was 42.11 min (RSD 1.7%; $n = 3$). A peak, indicating hydrocortisone, was not found in this electropherogram. In principle, prednisolone and hydrocortisone could have

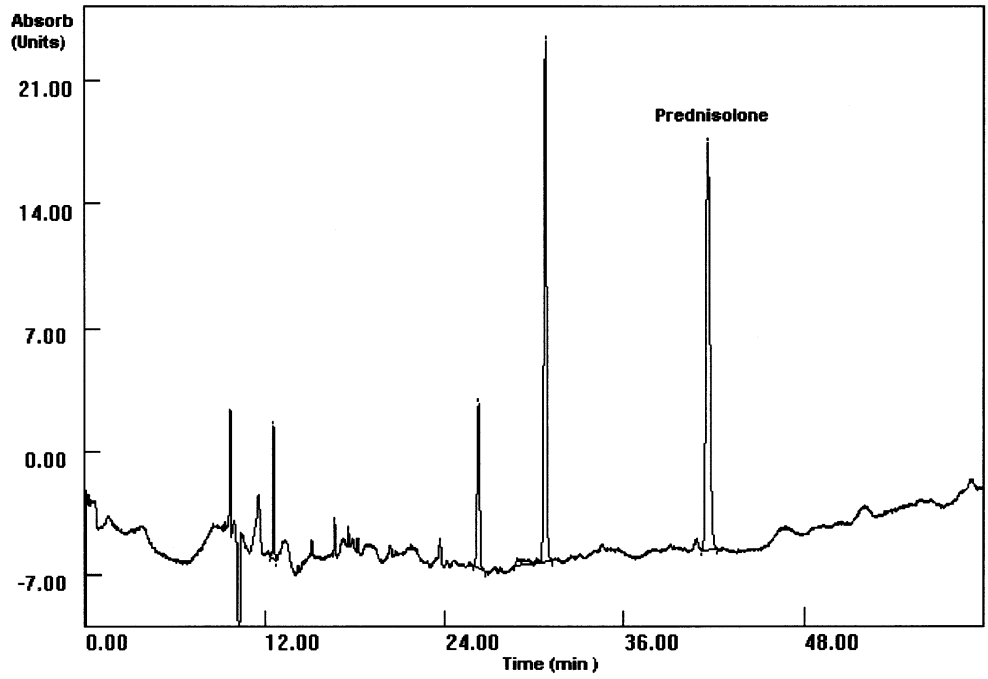

Fig. 3. Electropherogram of renal transplant's serum, treated with 500 mg Solu-Decortin H®, analysed in buffer II. Other conditions mentioned in text. Peak indicating prednisolone besides other unidentified substance peaks.

been separated in buffer II [19] in case of a serum level of hydrocortisone higher than the limit of detection (250 ng ml^{-1}).

Interference was found neither with prednisdone nor cortisone using one of the two buffers.

3.3. Quantitative determination of prednisolone using a calibration curve

Prednisolone levels in spiked serum and some patient serum specimen were calculated using peak heights and external standard calibration with calibration samples for each specimen. The calibration curve was linear in the range of expected serum levels (500 ng–4 μg ml^{-1}). The correlation coefficient (r) was calculated to 0.990. An example of a calibration curve is given in Table 1 with the migration times and peak heights of calibration samples. The limit of quantitation of prednisolone in serum was found to be 500 ng ml^{-1} with a relative signal to noise ratio of 3:1. The limit of detection was 250 ng ml^{-1}. As an example the concentration of prednisolone in the specimen, the electropherogram of which is shown in Fig. 2, was calculated to 3.3 μg ml^{-1} using the corresponding calibration curve.

Table 1
Migration times and peak heights of calibration samples (prednisolone in methanol) as example of a typical calibration curve

Conc. (μg ml^{-1})	Migration time [t_m] (min)	RSD of [t_m] (%)	Peak height [h] (units)	RSD of [h] (%)	n
0.5	6.85	7.1	2.02	20.5	4
1.0	6.42	0.6	2.96	19.0	4
2.0	6.85	1.8	7.60	16.5	4
3.0	6.45	2.2	12.95	15.3	4
4.0	6.10	1.7	14.99	6.3	4

4. Conclusion

The paper describes the method development for quantitative determination of prednisolone in human serum with MECC. The application of the method is promising for the measurement of prednisolone in serum of intensive care patients undergoing a high-dose steroid treatment. The extraction procedure is fast and easy to perform and does not require >1 h. Linearity of the calibration curve was good, reproducibility of the peak heights was sufficient. In some preliminary studies serum levels of prednisolone after infusion of 500 mg prednisolonehydrogenesuccinate were monitored up to 6 h. Nevertheless, prednisolone serum levels of patients undergoing low-dose steroid treatment (10 mg prednisone once a day per os) could so far not be measured with this method due to low sensitivity of UV-detection. The detection limit and the reproducibility of peak heights might significantly be enhanced using a 'z-shaped' capillary flow cell for UV detection, which is now under investigation.

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