ELSEVIER

Journal of Chromatography B, 672 (1995) 33–44

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Biocompatible sample pretreatment for immunochemical techniques using micellar liquid chromatography for separation of corticosteroids

Ulf Lövgren[a,*], Malin Johansson[b], Karin Kronkvist[b], Lars-Erik Edholm[a]

[a]*Department of Bioanalytical Chemistry, Astra Draco AB, P.O. Box 34, S-221 00 Lund, Sweden*
[b]*Department of Analytical Chemistry, University of Lund, P.O. Box 124, S-221 00 Lund, Sweden*

First received 26 May 1994; revised manuscript received 27 April 1995; accepted 9 May 1995

Abstract

Micellar liquid chromatography (MLC) using Tween 20 as surfactant was evaluated as a biocompatible sample pretreatment preceding immunoassay in order to obtain an increased selectivity of the assay and a simplification of the sample pretreatment procedure. Different stationary phases and chromatographic conditions were studied for the separation of budesonide and cortisol and some steroids known to interfere in immunoassay of these compounds. The separation was dependent on several parameters, for example, temperature, the concentration of Tween 20, pH and ionic strength of the mobile phase, and nature of the stationary phase. A precolumn venting system was used, which allowed for 140 direct injections of 25 μl of human blood plasma, without loss of chromatographic performance. Results obtained from the coupling of MLC to an immunoassay for cortisol illustrates the selectivity which can be obtained, and that simplification of the sample pretreatment is possible using this technique.

1. Introduction

Due to the potency and way of administration of many drugs or drug candidates, modern pharmaceutical research needs access to analytical methods where concentrations at picomolar levels or less can be measured [1,2]. Immunoassay is often used for these purposes because it is one of the most powerful techniques for determination of low concentrations of analytes in biosamples. However, antibodies used in immunological techniques often show cross-reactivity towards compounds of similar structure as the analyte in the sample. This limits accurate determination of the analyte. If the analyte concentration in the sample is much lower than that of the cross-reacting compounds, the assay errors may be large. In drug analysis and in early phases of drug development special care must be taken to possible cross-reactivity towards metabolites of similar structure. In the latter case this is of special importance as not all metabolites might be known. Monoclonal antibodies are sometimes used to improve this situation, but this is not a general solution to the problem. As an example, commercially available cortisol antibodies may have a substantial cross-reactivity towards prednisolone and cortisone.

* Corresponding author.

0378-4347/95/$09.50
SSDI 0378-4347(95)00198-0

To increase the selectivity, column liquid chromatography (CLC) is often used in combination with immunoassays. After separation, the fraction containing the analyte is collected and the eluent is usually evaporated to remove organic modifiers. The sample is then dissolved in a buffer compatible with the immunoassay. This time-consuming procedure is hard to automate and may lead to erroneous results due to a decrease in recovery and a potential risk for contamination.

It would be desirable to combine the inherent sensitivity of immunoassays with an increase of the selectivity and simplification of the sample preparation, utilizing direct injection of the biological matrix into the CLC system and direct transfer of the analyte for immunochemical detection. Direct injection of biological fluids in CLC can be obtained in a number of ways, for example, with the use of a precolumn with similar bonding characteristics as the analytical column [3]. A more recent approach is to use the so-called internal surface reversed-phase columns, or restricted access columns, where the porous solid support particles have a hydrophobic phase in the pores, not accessible for proteins [4,5]. In these applications, the mobile phase usually contains a substantial amount of organic modifier, which may have a negative effect on immunochemical and enzymatical reactions. Micellar liquid chromatography (MLC), however, is a technique that has been used for direct injections of biological fluids without the need for organic modifiers [6–9]. Micelles formed by the surfactant in the mobile phase solubilize the proteins and prevent them from denaturation and adsorption on the solid support [6].

The aim of this work was to investigate the possibility of using MLC as a sample pretreatment technique with direct injection of plasma samples, and separation of cross-reacting compounds coupled to an immunoassay for steroids. Tween 20 was chosen as micellar agent in the mobile phase, due to its characteristics of preventing non-specific adsorption in immunoassays [10–12] and its low influence on the activity of many enzymes. Surfactants are also often compatible with various detection principles, e.g. UV, fluorescence [13–15] and electrochemical detection [1].

Chromatographic characteristics using different stationary phases were investigated, as well as the effect on selectivity for the separation of some common corticosteroids at different concentrations of the micellar agent. Budesonide, a synthetic corticosteroid drug mainly used in the treatment of asthma, cortisol and some steroids known to interfere in immunoassay of these compounds were used as model compounds. Cortisol is a steroid hormone, commonly assayed in clinical practice in plasma or urine samples by means of immunoassay. Cross-reaction of cortisol antibodies towards endogenous corticosteroids and synthetic steroid drugs, such as prednisolone, may be severe. Several systems based on CLC, with different detection principles, have been developed to overcome this problem [16–18]. These approaches use mobile phases not compatible with immunoassays. In this work the potential of coupling MLC to immunochemical detection was evaluated using an immunoassay for the determination of low concentrations of steroids, developed in our laboratory [2].

2. Experimental

2.1. Apparatus

In the single column system the mobile phase was delivered by an LKB 2150 pump (LKB-Pharmacia Biotechnology, Bromma, Sweden) and the samples, 40 μM of each steroid, were injected using a Valco six-port injection valve (Valco Instruments, Houston, TX, USA), with a loop volume of 25 μl. The detector used for monitoring the steroids was an LKB 2151 UV monitor (LKB-Pharmacia Biotechnology) and an LKB water bath was used to thermostat the analytical column. All connecting capillaries were made of stainless steel with an inner diameter of 0.25 mm, and the flow-rate was 0.5 ml/min unless otherwise stated. Columns used in the MLC system were of the dimension 100×3.0 mm and were packed with silica-based 5 μm Nucleosil C_1, C_{18} or aminopropyl (Machery-

Nagel, Düren, Germany) or 3-μm Apex II aminopropyl (Jones Chromatography, Littleton, CO, USA). A polymer-coated 3-μm Spherisorb C_1, pH-stable, material (Phase Sep, Norwalk, CT, USA) was also used.

Common for both precolumn venting systems were the two LKB 2150 pumps, a Waters M440 UV detector (Waters, Milford, MA, USA) and a Varian 9090 autosampler (Varian Instruments, Sunnyvale, CA, USA), with a loop volume of 50 μl, for the direct injections of plasma samples. For the budesonide separation, an Apex II aminopropyl column (50 × 2.1 mm) was used as a precolumn, connected to a Valco six-port injection valve, and a Spherisorb C_1, pH-stable, column (50 × 2.1 mm) was used for the final separation. Another Valco six-port valve was used as a mobile phase selector. In the system for cortisol analysis, both columns were packed with Spherisorb C_1 pH-stable. The dimensions were 30 × 2.1 mm for the precolumn and 150 × 2.1 mm for the analytical column. After the precolumn, 0.1 mm I.D. stainless-steel connecting capillaries were used to keep the dispersion low in the system.

Microtitre plate readings were performed with a Multiscan MCC/340 type 347 (Labsystems, Helsinki, Finland) equipped with a 492-nm filter, and the immunoassay results were evaluated using Multicalc, a computer program for immunoassay measurements (Pharmacia-Wallac, Turku, Finland). Washing of microtitre plates was performed by an Ultrawash II MA 56 (Dynatech Laboratories, Chantilly, VI, USA), and dispensing of enzyme substrate and amplifier solution was carried out by a Multidrop type 831 automatic dispenser (Labsystems).

2.2. Chemicals

Budesonide was synthesized at Astra Draco AB. Cortisol, 11-deoxycortisol, progesterone, prednisolone and Tween 20 was purchased from Sigma Chemicals (St. Louis, MO, USA). The structures of the steroids are shown in Fig. 1. All buffer components were of p.a. grade. The composition and pH of the buffers used in the experiments are given in the legends to the figures.

The following chemicals were used in the cortisol immunoassay. Monoclonal antibodies towards cortisol, from mouse (Pierce, Rockfort, IL, USA), cortisol–alkaline phosphatase conjugate from Fitzgerald (Concord, MA, USA),

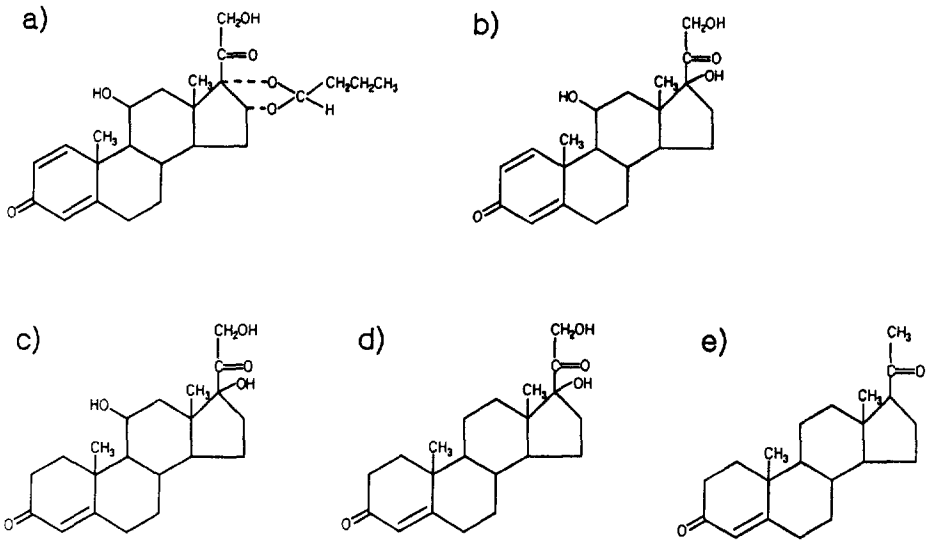

Fig. 1. Molecular structure of the synthetic steroids (a) budesonide and (b) prednisolone, and the endogenous steroids (c) cortisol, (d) 11-deoxycortisol and (e) progesterone, used as model substances.

Protein G from *Streptococcus sp.* and bovine serum albumin, BSA (fraction V), was obtained from Sigma Chemicals. AMPAK enzyme amplification kit containing substrate solution (NADPH), amplifier solution (alcohol dehydrogenase, diaphorase, ethanol and INT-violet) from DAKO Diagnostics (Cambridgeshire, UK).

2.3. Preparation of plasma samples

Human blood was drawn into Venoject vacuum tubes containing sodium heparin and after centrifugation at 1000 *g* for 10 min the plasma fraction was collected and stored at −20°C. Frozen human blood plasma was thawed and centrifuged for 10 min at 2500 *g* in order to remove any precipitated material. The supernatant was spiked with steroids (100 μM of each) and mixed with an equal volume of 1 *M* glycine buffer with 0.2% Tween 20, pH 2.5 for budesonide samples and 3.0 for cortisol samples. The samples were once again centrifuged, for 10 min at 2500 *g*, to remove any solid substance.

In the cortisol analysis, human blood plasma treated with activated charcoal was used as a blank matrix for standard sample preparation. This was done in order to remove endogenous steroids from the plasma. Activated charcoal (0.2 g) was added to 10 ml plasma and shaken for 60 min. The activated charcoal was then separated from the purified plasma by centrifugation at 1000 *g* for 30 min. The plasma was transferred to a new test tube and centrifuged again. This procedure was repeated several times until no charcoal could be observed in the plasma.

2.4. Immunoassay procedure

The enzyme-amplified ELISA for cortisol was carried out according to Lövgren et al. [2] with some modifications. Microtitre plates were coated with protein G (10 μg/ml in 0.05 *M* sodium carbonate buffer pH 9.6) overnight, to obtain a surface in the wells that would bind cortisol antibodies in a reproducible way. After blocking with BSA and Tween 20, and washing the plates, 150 μl of each sample was transferred directly from the collection tubes in the MLC system to wells on the plate using a Socorex eight-channel pipette (Renens, Switzerland). Aliquots of 30 μl cortisol–alkaline phosphatase conjugate (1:50 000) and 30 μl monoclonal antibodies towards cortisol (2.5 ng/ml) were then added to each well. Both the antibody and enzyme conjugate solutions were diluted in 0.05 *M* Tris-HNO_3, pH 7.3, with 0.15 *M* NaCl, 0.1% (w/v) BSA and 0.1% (v/v) Tween 20. After incubation overnight at room temperature the plates were washed four times and 75 μl AMPAK substrate solution was added. Incubation in room temperature in the dark for 30 min was followed by an addition of 75 μl AMPAK amplifier solution. After an incubation of 20 min, the dark red colour of formazan was monitored.

3. Results and discussion

To study the chromatographic characteristics in CLC using Tween 20 as a moderator, budesonide, cortisol and some endogenous steroids were chosen. The endogenous steroids are known to cross-react in immunoassays of cortisol. The retention behaviour was studied for different stationary and mobile phases. When the surfactant concentration was limited to 0.1% (v/v), Nucleosil C_1 and C_{18} gave very long retention times for most of the model substances. Due to the lower retention factors, *k*, obtained with the aminopropyl materials, a higher sample throughput was possible with maintained separation efficiency. Both Nucleosil and Apex II aminopropyl gave similar chromatographic characteristics of the steroids, but the efficiency on Apex II was higher mainly due to the smaller particle size. The separation of the steroids, used as model substances, on an aminopropyl column is shown in Fig. 2. It was observed that the column temperature had a significant effect on column efficiency and retention factor (Fig. 3). Based on these observations, 40°C was chosen as a suitable temperature for further experiments.

According to Refs. [19,20] the retention factor for an analyte is dependent on the micellar concentration in the mobile phase, and the relation can be described by the following equation

$$1/k = (K_{AM}/\Phi K_{SW})[M] + 1/\Phi K_{SW} \quad (1)$$

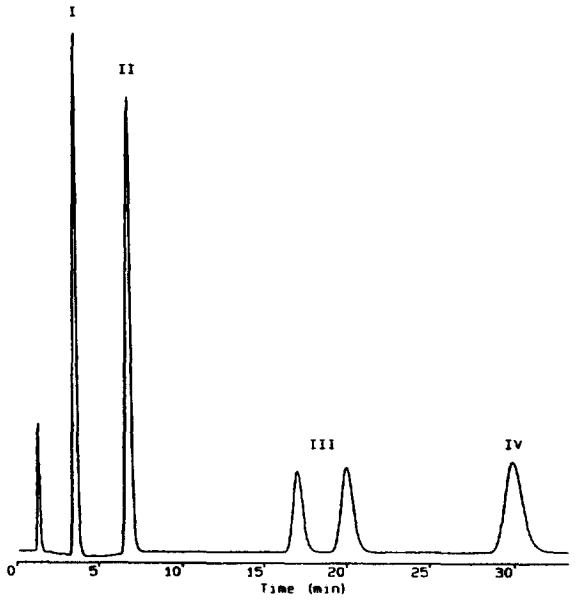

Fig. 2. Chromatogram of the separation of budesonide from endogenous steroids on an aminopropyl column (3 μm, Apex II; 100 × 3.0 mm), thermostated at 40°C using 0.01 *M* glycine buffer pH 2.5 with 0.1% Tween 20 as mobile phase, and a flow-rate of 0.5 ml/min. Peaks: I = cortisol, II = 11-deoxycortisol, III = epimer 22S and 22R of budesonide, and IV = progesterone. A volume of 25 μl was injected of the sample, consisting of 40 μM of each steroid.

where K_{AM} is the binding constant for the analyte–micelle complex and K_{SW} the distribution constant between stationary phase and water. $[M]$ is the total surfactant concentration in the mobile phase minus the critical micellar concentration, CMC, and Φ is the ratio of the volume stationary phase to mobile phase in the column.

According to Eq. 1 a straight line should be obtained when $1/k$ is plotted versus the micelle concentration [19–21]. In Fig. 4, this behaviour is shown for some steroids separated on the Nucleosil aminopropyl column. Calculated values of K_{AM}, determined from the slope and the intercept in the plot, are shown in Table 1. The positive values found represent micelle binding properties where the largest value was obtained for progesterone and the smallest for cortisol. In practice, this means that the surfactant concentration has only a small influence on the retention of cortisol. Nevertheless, the surfactant plays an important role in preventing protein adsorption on the stationary phase, and the retention may be affected further by selection of stationary phase and temperature. It is evident from Fig. 4 that the concentration of Tween 20 is

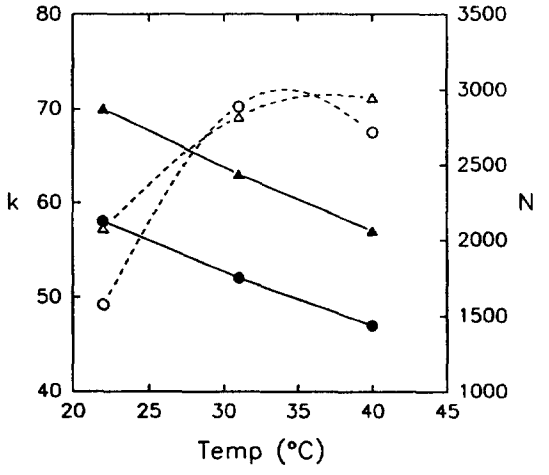

Fig. 3. Temperature-dependence of retention factor and plate number (dashed line) on an aminopropyl column (5 μm, Nucleosil; 100 × 3.0 mm). Mobile phase was 10 m*M* acetate buffer with 0.1% Tween 20, pH 4.0; ● represents the 22S, and ▲ the 22R epimer of budesonide. The budesonide concentration was 40 μM and 25 μl was injected.

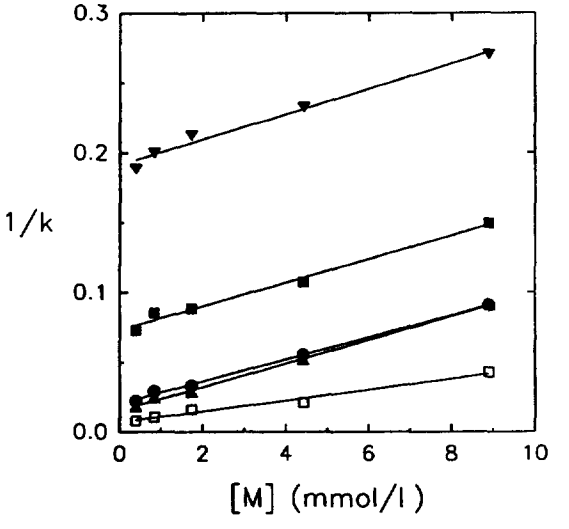

Fig. 4. Plot of $1/k$ versus micellized surfactant concentration for an aminopropyl column (5 μm, Nucleosil; 100 × 3 mm) with 10 m*M* acetate buffer, pH 4.0; ▼, cortisol; ■, 11-deoxycortisol; ● and ▲, the 22S and 22R epimers of budesonide, respectively, and □, progesterone. Concentration of steroids and injection volume as in Fig. 2.

Table 1
K_{AM} values for the steroids (l/mmol)

Steroid	K_{AM} pH 4.0 aminopropyl (Nucleosil)	K_{AM} pH 6.0 aminopropyl (Nucleosil)	K_{AM} pH-stable C_1 (Spherisorb)
Cortisol	0.05		
11-Deoxycortisol	0.11		
Budesonide epimer 22S	0.38	0.91	0.29
Budesonide epimer 22R	0.54	1.47	0.40
Progesterone	0.55		

important to optimize the retention and selectivity of the different steroids. However, in the immunoassay it is common to keep the concentration of Tween 20 at about 0.05% [22].

With the aminopropyl column, k for budesonide increased rapidly with pH of the mobile phase (Fig. 5). As seen in Table 1, K_{AM} for budesonide also varied with pH. Since the surfactant and the analytes are non-ionic, and the charge of the amino groups on the stationary phase is very little affected in the lowest part of the studied pH-range, the pH effect can probably

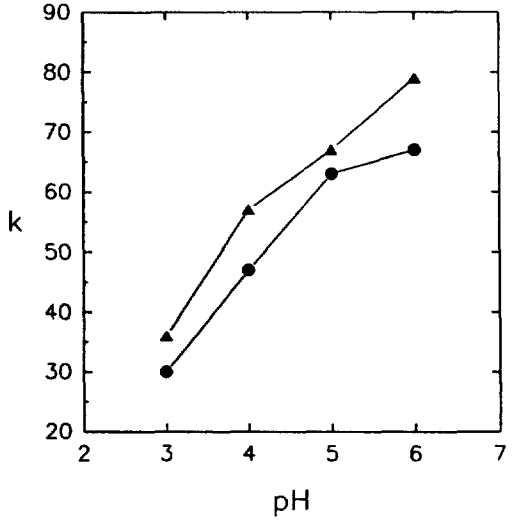

Fig. 5. pH-dependence of the retention of budesonide on an aminopropyl column (5 μm, Nucleosil; 100 × 3 mm) using 10 m*M* acetate buffer with 0.1% Tween 20, pH 4.0 as mobile phase; ● is the 22S and ▲ is the 22R epimer. Concentration of steroids and injection volume as in Fig. 2.

be ascribed to the acidity of residual silanol groups on the stationary phase. It has been reported that Tween 40 and some other surfactants adsorb strongly to silanol groups [23,24]. The degree of adsorption may be influenced by the pH in the mobile phase. Also the ionic strength had an effect on the retention for budesonide, where an increased concentration of $NaNO_3$ resulted in a linear increase of k between 0 and at least 200 m*M*. In this range k increased from 3 to about 20 for both epimers.

For non-ionic detergents the time to reach equilibrium in the column may be very long [25]. With the aminopropyl column the equilibration took several hours, and even after that time a small continuous increase in the retention time of the steroids could be seen (0.2% per hour). This is probably due to the well known degradation of the stationary phase in water solution, resulting in an increase in number of silanol groups which may adsorb Tween 20. Packing materials where the silica backbone is protected from the mobile phase were therefore chosen for further investigations.

The columns packed with polymer-coated silica material were equilibrated much faster than the silica-based, and no drift in retention could be seen when injecting the analytes dissolved in mobile phase. Also in this case an increase in temperature increased the plate number of the column, but the k-values increased with temperature contrary to the behaviour with the aminopropyl column (Fig. 6). In order to compare the data obtained with this column with data from the aminopropyl column, the separations were performed at 40°C. For the polymer-coated C_1 column both the K_{AM} value and the retention factor remained constant for budesonide in the pH range 2.5–4. The ionic strength had only little effect on the retention factor with this column, compared to the silica-based aminopropyl column.

Although organic solvents should be avoided in the mobile phase if the separation system is to be compatible with immunoassays, small amounts of organic modifiers may increase the efficiency in MLC. Some of this effect may probably be ascribed to a reduction in the

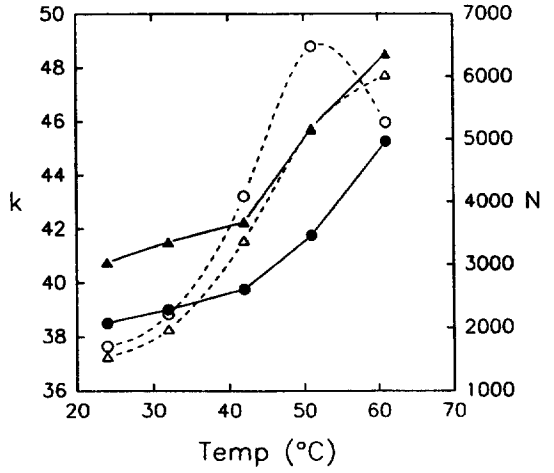

Fig. 6. Temperature-dependence of retention factor and plate number (dashed line) for the (●) 22S and (▲) 22R epimers of budesonide on a C_1 column (3 μm, Spherisorb; 100 × 3 mm) with 5 m*M* glycine buffer, 0.5% Tween 20, pH 2.5. Concentration of steroids and injection volume as in Fig. 2.

amount of surfactant adsorbed on the stationary phase [26,27]. Concentrations of 1-propanol up to about 3% in the mobile phase resulted in a substantial increase of the plate number (Fig. 7). At higher concentrations, the efficiency was rapidly lost possibly due to changed micellar conditions [25].

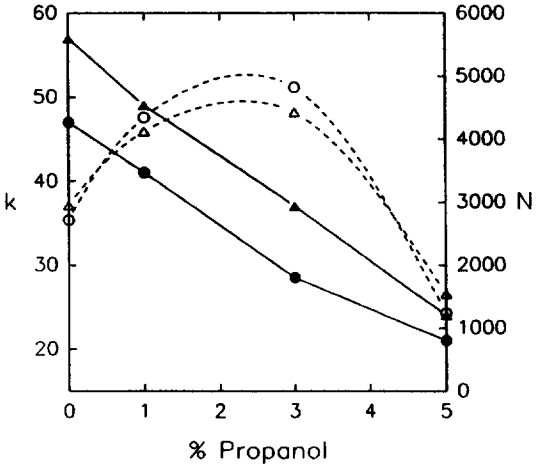

Fig. 7. Influence of propanol concentration on retention factor and plate number (dashed line) for the (●) 22S and (▲) 22R epimers of budesonide. Stationary phase was aminopropyl (5 μm, Nucleosil; 100 × 3 mm) and mobile phase 10 m*M* acetate buffer with 0.1% Tween 20, pH 4.0 and an addition of 0–5% 1-propanol. Concentration of steroids and injection volume as in Fig. 2.

3.1. Plasma samples

It has been reported that direct injection of serum and plasma samples in CLC is possible with the use of surfactants in the mobile phase, due to solubilization of proteins [6,8]. However, some late eluting plasma components, such as proteins and lipids, may still be retained on the column, resulting in a decrease in efficiency of the chromatographic system. The degree of efficiency loss will depend on several factors, for example the degree of protein binding of the analyte.

Direct injections of human blood plasma, spiked with budesonide, were performed using 0.1% Tween 20 in the mobile phase. With the aminopropyl column the retention time increased with about 0.2% per hour, and after 37 injections the plate number had decreased with about 35%. The efficiency of the column was, however, retained after a 1-h rinse of the column with mobile phase.

A 65% decrease in efficiency was observed for budesonide on the polymer-coated packing material, Spherisorb C_1 pH-stable, after 11 injections (25 μl each). To overcome this effect a precolumn venting system was used as shown in Fig. 8. The precolumn was chosen so that the analyte was trapped while major plasma components, seen with the UV detector, passed the column to waste. The valve was switched and the analyte was eluted from the trap column into the analytical column. For budesonide an addition of 1-propanol to the mobile phase (3% v/v) was used to decrease the retention volume. At a certain time, when the sample zone was in the end of the analytical column (see valve position table, Fig. 8), valve 1 was switched to remove the propanol from the flow in the analytical column obtaining biocompatible conditions before collecting the analyte fraction. Chromatograms obtained for plasma samples of budesonide at concentrations of 80 μM are shown in Fig. 9a. In Fig. 9b the repeatability of the procedure is visualized for budesonide showing that only a slight change is observed in the chromatographic characteristics after 140 injections of human blood plasma samples. It should be noted that for practical reasons UV detection was used in

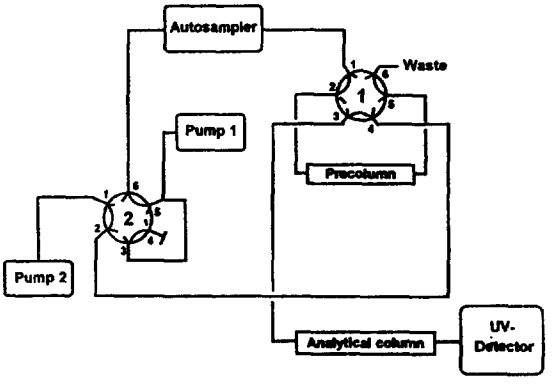

Table of valve positions:

Time [min]	Valve 1	Valve 2
0.0	A	A
1.4	B	A
5.0	A	A
14.0	A	B
30.0	A	A

Fig. 8. The precolumn venting system used for budesonide analysis with direct injection of blood plasma. The precolumn (30 × 2.1 mm) was packed with Apex II aminopropyl and the analytical column (50 × 2.1 mm) was packed with Spherisorb C_1 pH-stable. Valve position A represents the flow paths 1–2, 3–4, 5–6, and position B the flow paths 1–6, 2–3, 4–5.

the above studies. The concentration of budesonide obtained in clinical practise is much lower and the observations made may therefore be different at these concentrations.

3.2. Coupling to immunoassay

Micellar liquid chromatography can be coupled to immunological detection systems in different ways. One way is to collect the analyte fraction using a time-programmed fraction collector connected to the MLC system. After adjustment of the pH and surfactant concentration, if needed, the collected fractions can be transferred directly to a microtitre plate or to immunoassay test tubes without any time-consuming evaporation step.

In this work, plasma samples were pretreated using MLC to separate cortisol from other steroids, and the cortisol was then quantitated by means of enzyme immunoassay. The precolumn venting system, shown in Fig. 10, is a modification of the system used for budesonide. In order to transfer collected fractions directly to the microtitre plates, without any adjustment of the pH, the mobile phase used in this application was a 5 m*M* Tris-HNO_3 buffer, pH 7.3 with 0.15 *M* $NaNO_3$ and 0.1% (v/v) Tween 20. Instead of trapping the analyte on the precolumn and using backflush injection into the analytical column, the sample was injected into the precolumn, where the unretained fraction was distributed to waste. Before elution of cortisol from the precolumn, the analytical column was introduced in the flow by switching valve 2. Valve 1 was switched to position B (see Fig. 10) after complete elution of cortisol from the precolumn, in order to wash the column in backflush mode until the next injection of sample.

Fig. 11a shows the separation of cortisone, cortisol, prednisolone and 11-deoxycortisol on the precolumn. The heartcut is marked with dashed lines. The separation through the entire system is shown in Fig. 11b.

Plasma samples, treated with activated charcoal, were spiked with cortisol, run through the precolumn venting system and quantitated by immunoassay. A calibration curve is shown in Fig. 12. The total recovery for an authentic cortisol control (484 n*M*) was estimated to 75%. This was done by comparing the obtained ELISA response from a plasma sample injected into the MLC system, with a pure reference solution without sample pretreatment.

The cortisol concentration of authentic plasma samples was determined with the above described method, and compared with results from a validated method based on a coupled column liquid chromatography system with UV detection [18] routinely used in our laboratory, where prednisolone is separated from cortisol in a cyclodextrin column (see Table 2). The deviations between the two compared methods can be explained by the imprecision of the ELISA determination.

The cross-reactivity of the monoclonal cortisol antibodies used in this work is stated by the manufacturer (Pierce) to be 100% towards cor-

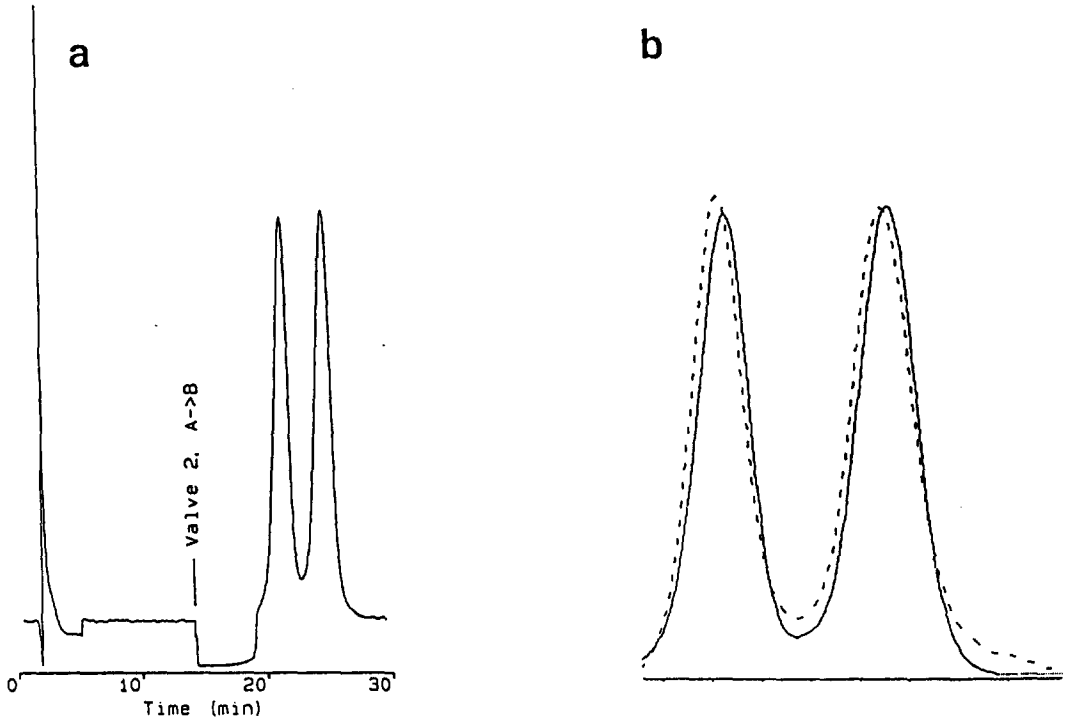

Fig. 9. (a) Chromatogram of a direct injected plasma sample, spiked with budesonide, using the precolumn venting system. Mobile phase composition was from pump 1: 10 m*M* glycine buffer, pH 2.5 with 0.1% Tween 20; from pump 2: 3% 1-propanol in 10 m*M* glycine buffer pH 2.5, with 0.1% Tween 20. The flow-rate was 0.5 ml/min and the columns, the same as shown in Fig. 8, were thermostated at 40°C; 50 μl of sample was injected with a concentration of 40 μM for each steroid. (b) Comparison of the chromatographic peak shape between the 1st (solid line) and the 140th injection (dashed line) of human blood plasma spiked with budesonide. All experimental details as in (a).

tisone, 45% towards prednisolone and 55% towards 11-deoxycortisol. If high concentrations of these steroids would be present in the sample, this antibody preparation, used in a direct immunoassay without preseparation, would give falsely high values of the cortisol concentration. The influence on the ELISA response from a plasma sample containing 476 n*M* prednisolone is shown in Fig. 13. The entire chromatogram was fractionated in 1-min fractions, which were all quantitated by ELISA measurements. The two large peaks are ascribed to cortisol and prednisolone; compare also with Fig. 11b where the same chromatographic conditions were used, but with UV detection. As seen in Table 2, the addition of prednisolone to the plasma sample does not result in a significantly different value for the measured cortisol concentration.

A miniaturization of the above CLC system would allow for direct transfer of the total sample volume containing the analyte to the wells of the microtitre plate. This would result in a further simplification of the above procedure and an increase in the lower limit of determination.

4. Conclusions

Micellar liquid chromatography using Tween 20 in the mobile phase has an interesting potential for sample pretreatment preceding detection by immunological techniques. Repeated direct injections of human plasma samples are possible when MLC is used in combination with a precolumn venting system.

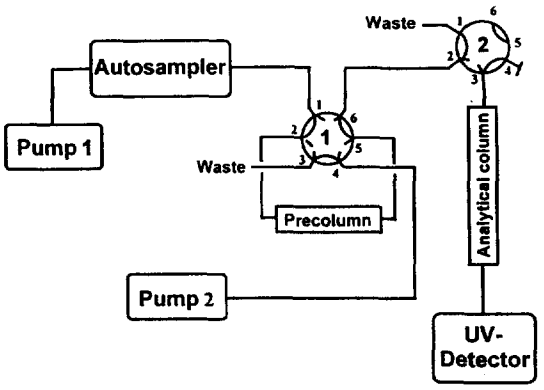

Table of valve positions:

Time [min]	Valve 1	Valve 2
0.0	A	A
3.0	A	B
6.0	B	B
34.0	A	A

Fig. 10. Precolumn venting system for cortisol analysis with direct injection of blood plasma. Both columns were packed with Spherisorb C_1 pH stable. Dimensions of the columns were 30×2.1 mm for the precolumn and 150×2.1 mm for the analytical column. Valve position A represents the flow paths 1–2, 3–4, 5–6, and position B represents the flow paths 1–6, 2–3, 4–5.

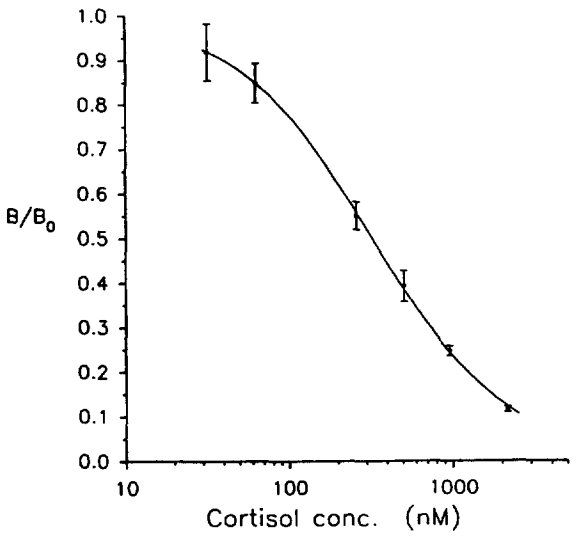

Fig. 12. Calibration curve for cortisol after direct injection of human blood plasma standards into the precolumn venting system with a subsequent competitive ELISA determination. The ELISA response is expressed as B/B_0, the normalized antibody-bound fraction of the enzyme conjugate. Each value is obtained from duplicate immunoassay readings and the standard deviation is visualized by the error bars.

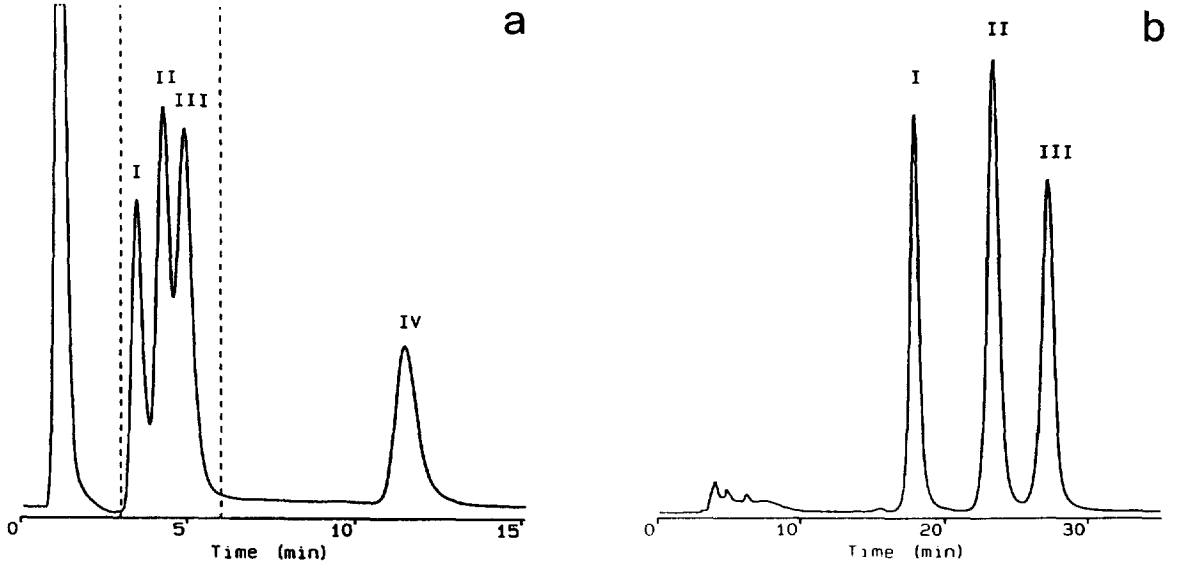

Fig. 11. (a) Chromatogram of the separation of (I) cortisone, (II) cortisol, (III) prednisolone and (IV) 11-deoxycortisol on the precolumn described in Fig. 10. (b) Chromatogram of the separation of above-mentioned steroids through the entire precolumn venting system. The mobile phase consisted of 0.1% Tween 20 in 5 m*M* Tris-HNO_3 with 0.15 *M* $NaNO_3$, pH 7.3. The flow-rate was 0.2 ml/min.

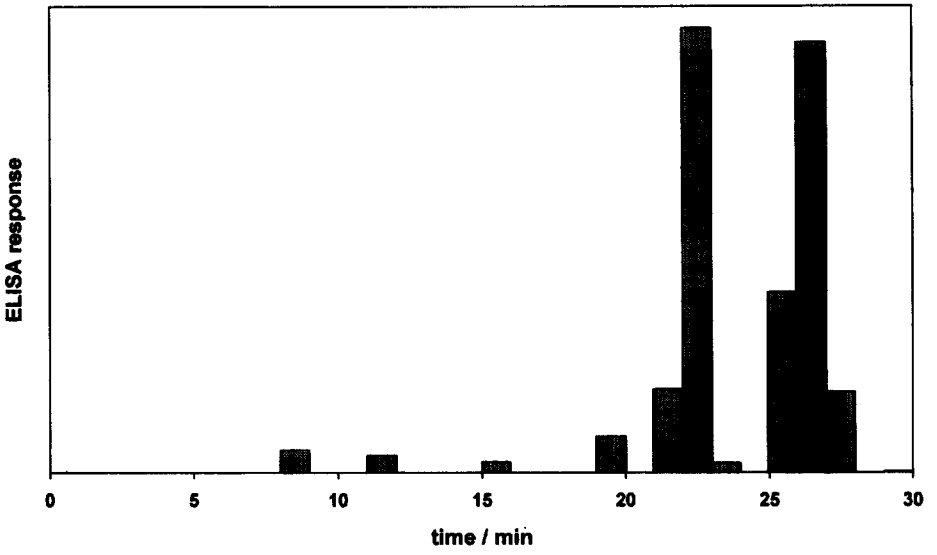

Fig. 13. A fractionated chromatogram of an authentic plasma sample, spiked with prednisolone (476 n*M*). The amount of steroids in each fraction was determined by ELISA and expressed as equivalent cortisol amount.

For the separation of the model compounds different types of packing materials can be used, but rather polar stationary phases must be used for hydrophobic analytes to avoid long retention times. Polymer-coated materials are recommended to overcome problems due to silanol groups which give rise to slow equilibration, an effect further amplified by slow degradation of the stationary phase.

For the polymer-coated packing material, several parameters have an effect on retention and efficiency. Most pronounced effects were obtained for the concentration of Tween 20 and the temperature. For the neutral analytes investigated in this work, neither pH nor the ionic strength had any significant effect on the separation parameters, probably due to the absence of silanol groups.

Laborious sample handling procedures for transfer of the analyte for detection by immunoassay can be obviated when using MLC with a mobile phase compatible with the immunoassay.

Table 2
Determination of cortisol in authentic plasma samples; single injections

Sample	Measured cortisol concentration (n*M*)	
	MLC + ELISA	CCLC + UV[a]
Control high	451	484
Control low	222	202
Control low spiked with prednisolone (476 n*M*)	179	

[a] A validated coupled column liquid chromatography system routinely used in our laboratory to analyse cortisol without interference from prednisolone [9].

Acknowledgement

Ms. Pia Jönsson is gratefully acknowledged for performing the cortisol analysis with the validated routine method, and for valuable discussions about activated charcoal treatment of plasma.

References

[1] K. Kronkvist, U. Lövgren, L.-E. Edholm and G. Johansson, J. Pharm. Biomed. Anal., 11 (1993) 459.
[2] U. Lövgren, K. Kronkvist, G. Johansson and L.-E. Edholm, Anal. Chim. Acta, 288 (1994) 227.

[3] D. Westerlund, Chromatographia, 24 (1987) 155.
[4] T.C. Pinkerton, J. Chromatogr., 544 (1991) 13.
[5] M. Beth, K.K. Unger, M.P. Tsyurupa and V.A. Davankov, Chromatographia, 36 (1993) 351.
[6] F.J. DeLuccia, M. Arunyanart and L.J. Cline Love, Anal. Chem., 57 (1985) 1564.
[7] L.J. Cline Love, S. Zibas, J. Noroski and M. Arunyanart, J. Pharm. Biomed. Appl., 3 (1985) 511.
[8] J. Haginaka, J. Wakai, H. Yasuda and T. Nakagawa, Anal. Chem., 59 (1987) 2732.
[9] I. Carretero, M. Maldonado, J.J. Laserna, E. Bonet and G. Ramis Ramos, Anal. Chim. Acta, 259 (1992) 203.
[10] S.H. Jenkins, W.R. Heineman and H.B. Halsall, Anal. Biochem., 168 (198) 292.
[11] K. Mohammad and A. Esen, J. Immunol. Methods, 144 (1991) 19.
[12] Surfactants, Product information from Serva Finebiochemica.
[13] F.G. Sanchez, A.L.R. Rubio, C.C. Blanco and M. Hernandez, Anal. Chim. Acta, 205 (1988) 139.
[14] S. Igarashi and W.L. Hinze, Anal. Chem., 60 (1988) 446.
[15] L.B. McGown, in T.T. Ngo (Editor), Nonisotopic Immunoassay, Plenum Press, New York, NY, 1988, p. 245.
[16] E. Canalis, G.E. Reardon and A.M. Caldarella, Clin. Chem., 57 (1985) 1564.
[17] J.H.M. van den Berg, C.H.R. Mol, R.S. Deelder and J.H.H. Thijssen, Clin. Chim. Acta, 78 (1977) 165.
[18] L.-E. Edholm, A. Blomgren, C. Roos and L. Ögren, Chemometrics and intelligent laboratory systems, Lab. Inf. Manage., 17 (1992) 233.
[19] D.W. Armstrong and F. Nome, Anal. Chem., 53 (1981) 1662.
[20] M. Arunyanart and L.J. Cline Love, Anal. Chem., 56 (1984) 1557.
[21] M.J.M. Hernandez and M.J.G. Alvarez-Coque, Analyst, 117 (1992) 831.
[22] P. Tijssen, in R.H. Burdon and P.H. van Knippenberg (Editors), Practice and Theory of Enzyme Immunoassays, Elsevier, Amsterdam, 1985, p. 331.
[23] A. Berthod, I. Girard and C. Gonnet, Anal. Chem., 58 (1986) 1359.
[24] R.A. Wall, J. Chromatogr., 194 (1980) 353.
[25] M.F. Borgerding and W.L. Hinze, Anal. Chem., 57 (1985) 2183.
[26] M.F. Borgerding, R.L. Williams, W.L. Hinze and F.H. Quina, J. Liq. Chromatogr., 12 (1989) 1367.
[27] R. Bailey and R.M. Cassidy, Anal. Chem., 64 (1992) 2277.