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Determination of drugs in biosamples at picomolar concentrations using competitive ELISA with electrochemical detection: application to steroids

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Abstract: A competitive ELISA with electrochemical detection in a flow injection system (FIA) has been developed for determinations of the steroid drug budesonide in biological samples. Plasma samples were cleaned from interfering and cross-reacting compounds by two pretreatment steps consisting of a solid-phase extraction and a liquid chromatography fractionation. The enzyme label was alkaline phosphatase, which was used with *p*-aminophenyl phosphate (PAPP) as a substrate. The product, *p*-aminophenol, was detected electrochemically at a glassy carbon electrode at 250 mV (vs Ag/AgCl). The limited stability of both the substrate and the product influenced the performance of the method and had to be taken into account in the procedure by a normalization with time. Budesonide could be quantified in plasma samples down to 10 pM. The major sensitivity-limiting factor was the amperometric background response, probably due to spontaneous hydrolysis of PAPP to *p*-aminophenol.

Keywords: *Enzyme immunoassay; alkaline phosphatase; electrochemical detection; budesonide; blood plasma.*

Introduction

Modern pharmaceutical research needs access to analytical methods for determination of drugs and drug metabolites during all phases of product development. The sample matrices are biological and a particular task is the establishment of a pharmacokinetic profile by determining the absorption, distribution, metabolism and excretion of the drug. Modern drugs show ever increasing potency and determinations have therefore nowadays to be made in the nM–pM range. The drug concentrations may be even lower if it is administered locally or if it undergoes extensive metabolism.

One or more separation steps are often necessary before detection because of the complexity of biological matrices and the most commonly used separation technique in bioanalysis is column liquid chromatography (CLC). The analyte can either be detected as such or after derivatization [1]. Detection schemes utilizing electrochemistry [2], fluorescence [1] or chemiluminescence [1] have been successfully used in combination with CLC for a number of compounds of pharmaceutical and biochemical interest. More recently, mass spectrometers have become available as detectors in combination with CLC, showing great potential for determination of drugs at low concentrations [3]. Although the sensitivity obtainable using CLC in combination with selective detectors is promising, there are still very few applications in the literature describing determinations at picomolar concentrations.

Immunoassay is one of the most powerful analytical techniques for selective and sensitive detection in bioanalysis. Selectivity is high because of the antibody–antigen interactions and it should therefore be possible to obtain very low detection limits in theory [4]. In practice, however, there are few applications where the potential of immunoassays for determination at the low picomolar level have been explored for low molecular compounds. Some examples are given by refs 5–8.

Cross-reactivity towards similar compounds in the sample matrix can be expected, especially from endogenous compounds present in much higher concentrations. No visual information is available to indicate the presence of interfering compounds in immunoassays, in contrast to chromatography, and a sample pre-

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treatment step might therefore become necessary [9]. This is especially important in early stages of drug development where non-identified metabolites might cross-react.

Glucocorticosteroids are potent antiinflammatory agents and they are consequently widely used in the treatment of inflammatory disorders such as asthma [10]. The preferred route of drug administration is via inhalation of relatively small amounts of the drug resulting in a therapeutic drug concentration in the airways with plasma concentrations in the nM–pM range. Budesonide is a glucocorticosteroid that has been used in the inhalation treatment of asthma for more than 10 years and it is used as a model substance in the present study. Previously used methods for determinations of budesonide in plasma rely upon an LC–radioimmunoassay [11] and more recently on LC–mass spectrometry [12]. The latter allows for a limit of quantification of 100 pM, using 3 ml plasma.

In this work an enzyme-linked immunoassay (ELISA) in combination with flow injection analysis (FIA) and amperometric detection have been evaluated for use in bioanalysis of steroids.

Experimental

Apparatus

The *p*-aminophenol generated by the alkaline phosphatase catalysis was detected in a FIA-system consisting of an LKB 2150 HPLC-pump (Pharmacia LKB Biotechnology, Uppsala, Sweden) and an electric six-port injection valve (Valco Instruments Inc., Houston, TX, USA) with a loop volume of 25 μl. The detector was a thin-layer, BAS CC-4, flow-through cell (Bioanalytical Systems, West Lafayette, IN, USA) with a glassy carbon working electrode, an Ag/AgCl reference electrode (3 M NaCl) and an auxiliary electrode made of stainless steel. An Access*Chrom chromatography computer system v. 1.6 (Perkin–Elmer Nelson systems, Cupertino, CA, USA) was used for integration of the output signals from the BAS LC-4 potentiostat. Evaluations of the immunoassay results were made with 'Multicalc', a computer program for immunoassay measurements (Pharmacia–Wallac OY, Turku, Finland).

Materials

Budesonide [11β,21-dihydroxy-16α,17α-[22*R*,*S*]-propylmethylenedioxy pregna-1,4-diene-3,20-dione] and budesonide-21-hemisuccinate were synthesized by Astra Draco AB. Antiserum raised towards budesonide-21-hemisuccinate (from sheep) was obtained from the University of Surrey (UK) [13]. The IgG fraction was salted out and purified on a protein-G column (Pharmacia LKB Biotechnology, Uppsala, Sweden). *p*-Aminophenyl phosphate was synthesized by Astra Draco AB according to De Riemer [14].

Alkaline phosphatase (AP) E.C. 3.1.3.1 (from calf intestine), Tween 20 and bovine serum albumin (BSA) fraction V, were purchased from Sigma Chemical Company (St Louis, MO, USA). Kathon CG was obtained from Sjöstrand Kemi AB (Helsingborg, Sweden). All other chemicals were of p.a. grade.

Maxisorp microtitre plates were purchased from Nunc (Roskilde, Denmark). Ultrafiltration tubes were obtained from Amicon Division (W.R. Grace & Co., Beverly, MA, USA) and Bond-Elut columns came from Analytichem International (Harbor City, CA, USA).

Preparation of budesonide–alkaline phosphatase conjugate

Budesonide-21-hemisuccinate was coupled to AP using the mixed anhydride method [15]. A 1.5 mg mass of budesonide-21-hemisuccinate was dissolved in 100 μl dimethyl formamide and cooled to 10°C. A 2 μl volume of isobutyl chloroformate was added and the mixture was stirred for 30 min at 10°C. 1000 units of AP (0.18 mg) was diluted to 2 ml with a carbonate buffer (pH 9.2; 0.1 M). The enzyme solution was added drop-wise to the steroid derivative and the mixture was stirred for 4 h at 10°C and then overnight at 4°C. During this reaction the pH was maintained at 9.0. The resulting conjugate solution was ultrafiltered four times using Amicon tubes (cut-off 30 000 D), with 0.05% Tween 20 in the buffer and diluted to 2.0 ml with Tris–HCl buffer (pH 6.75; 0.1 M) containing 1 mM $MgCl_2$. Kathon CG, 50 μl l^{-1}, was added to the buffer to prevent bacterial growth.

After conjugation the enzyme activity was measured spectrophotometrically at 310 nm. It was found that 46% of the original enzyme activity was retained.

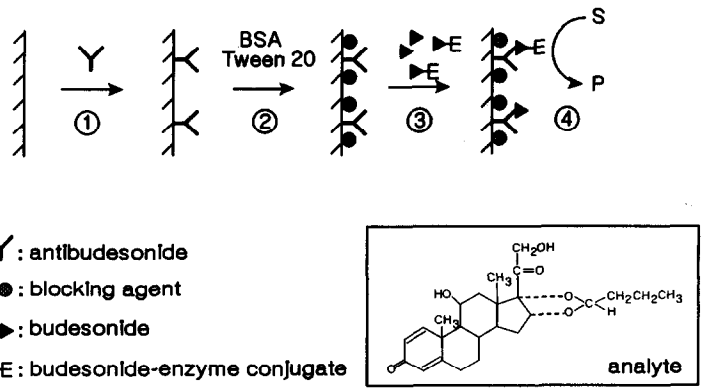

Figure 1
The ELISA procedure involving (1) coating the wells with antibudesonide, (2) blocking with BSA and Tween 20, (3) incubation with sample and enzyme conjugate; and (4) substrate incubation.

Buffers

The following buffers were used in the assay:

(A) 0.1 mM sodium acetate, adjusted to pH 5.0 with acetic acid;

(B) 0.05 M Tris(hydroxymethyl)aminomethane, 0.1% (w/v) BSA, 0.15 M NaCl and 0.05% (v/v) Tween 20. pH was adjusted to 7.3 with nitric acid;

(C) 0.1 M Tris–HNO_3, 1.0 mM $MgCl_2$ and 0.05% (v/v) Tween 20, pH 8.0;

(D) 0.1 M NaH_2PO_4, adjusted to pH 8.0 with NaOH; and

(E) A 5:2 mixture of buffer C and D.

Assay procedure

The competitive ELISA for budesonide was performed according to the scheme in Fig. 1. Microtitre plates were coated with 100 µl antibudesonide diluted (1:5000) in buffer A and incubated overnight at room temperature. The wells were blocked with 300 µl 2% (w/v) BSA and 0.05% (v/v) Tween 20 in buffer A and kept in room temperature for 2–3 h. Subsequently the wells were washed four times with buffer B.

Enzyme-conjugated steroid (1:10 000) and samples (or standards), diluted in buffer B (200 µl total volume), were added to each well and incubated over night in room temperature. They were then washed twice with buffer B (but without BSA). Freshly prepared PAPP (150 µl, 2 mM) in deaerated buffer C was added to the wells and incubated for 60 min in the dark. The enzyme reaction was then stopped by transferring 100 µl from each well to 40 µl buffer D. *p*-Aminophenol, the reaction product, was determined by duplicate injections of 25 µl aliquots into the flow system using buffer E as a carrier, i.e. the same buffer as that used for dissolving the samples.

Sample pretreatment

Samples of human blood plasma were pretreated according to the scheme in Fig. 2. Budesonide was added to plasma, centrifuged and purified by a solid-phase extraction using 500 mg C_{18} Bond-Elut columns. Before addition of the plasma sample (2.5 ml) the columns had been treated once with methanol and twice with deionized water. The columns were rinsed with 5 ml portions of water, with ammonium acetate buffer (pH 7.0; 5 mM) containing 35% methanol and with water again. Phase reversal was made with heptane and the budesonide was eluted in 5 ml 35% ethylacetate in heptane. These fractions were dried to completeness in a Savant 'Speed vac

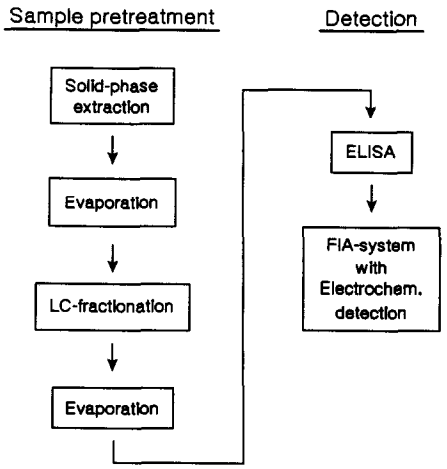

Figure 2
Scheme of sample pretreatment and detection procedure.

concentrator' (Savant Instruments Inc., Farmingdale, NY, USA).

The samples were dissolved in 350 μl formic acid (5 mM) containing 25% ethanol and 300 μl was injected into an LC-system with a 2.1 × 50 mm column packed with 3 μm C_{18} Nucleosil (Machery–Nagel, Düren, Germany), thermostatted to 40°C, and a Guard-Pak Resolve silica column (Waters, Millipore Corp., Milford, MA, USA). Since the samples were dissolved in a buffer containing less ethanol (25%) than the mobile phase (35%) a large sample could be injected due to peak compression. Fractions of 1 ml were collected with a flow rate of 0.5 ml min^{-1} and the fraction containing budesonide was dried and redissolved in 120 μl buffer B. Duplicate ELISA determinations were made by adding 40 μl to each of two wells on the microtitre plates.

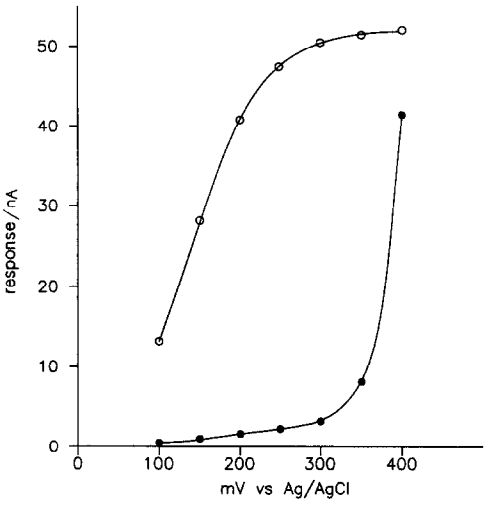

Figure 3
Hydrodynamic voltammograms of (○) 2 μM *p*-aminophenol and (●) 2 mM PAPP in buffer E.

Results and Discussion

The electrochemical detection

Alkaline phosphatase is one of the most commonly used enzyme labels in ELISA and the substrate is usually *p*-nitrophenyl phosphate when using spectrophotometrical detection. Phenyl phosphate has often been used as a substrate when the detection is made electrochemically, but the product, phenol, poses a number of problems. It is oxidized only at high applied electrode potentials, over +800 mV, and electrode fouling reactions decrease the sensitivity with time. *p*-Aminophenyl phosphate (PAPP) is reported to be dephosphorylated faster by the enzyme than *p*-nitrophenyl phosphate and phenyl phosphate [16, 17] and the electrochemical oxidation of the product, *p*-aminophenol, occurs at lower potentials. Interferences from matrix components and the size of the background currents are reduced at the lower potential and the sensitivity is thereby increased. PAPP has been used as a substrate in a number of electrochemical immunoassay detection schemes [17–19] and in electrochemical determinations of alkaline phosphatase [20].

p-Aminophenol has a linear response over a wide concentration range, with a detection limit of 5 nM or 0.1 pmoles (25 μl injected, $S/N = 2$). The cyclic voltammetry peak potential for oxidation of *p*-aminophenol is +60 mV and that of PAPP is +550 mV (vs Ag/AgCl) in buffer C. Figure 3 shows a comparison of the oxidation of *p*-aminophenol in amperometric mode with that of the substrate, PAPP, when the latter is present in a 1000-fold excess. This plot illustrates that the selectivity is substantial, but still not as good as desired.

Injections of a blank with PAPP, but without addition of *p*-aminophenol will produce a peak, which will be referred to as the FIA-blank below.

The current-voltage relation for an electrochemical reaction can be approximated by the Tafel equation at sufficiently high overpotentials, η. Mass transfer effects can be neglected if the current is less than 10% of the limiting current [21]. For positive overpotentials

$$\eta = \text{const. } 2.3\, RT(\log i)/(1 - \alpha)nF,$$

where n is the number of electrons in the reaction, α the transfer coefficient and the other quantities have their usual meaning. The logarithm of the current is plotted versus the applied potential in Fig. 4. A Tafel slope of 59 mV $decade^{-1}$ is obtained with $n = 2$ and $\alpha = 0.5$ and it is indicated as a straight line in the figure. It can be seen that there is a positive deviation from linearity in the form of a hump in the curve around 200 mV. Violations of the two assumptions made above will both cause negative deviations. This hump indicates, therefore, the presence of a small amount of a second redox couple.

One explanation is that an impurity is present in the preparation at a lower concen-

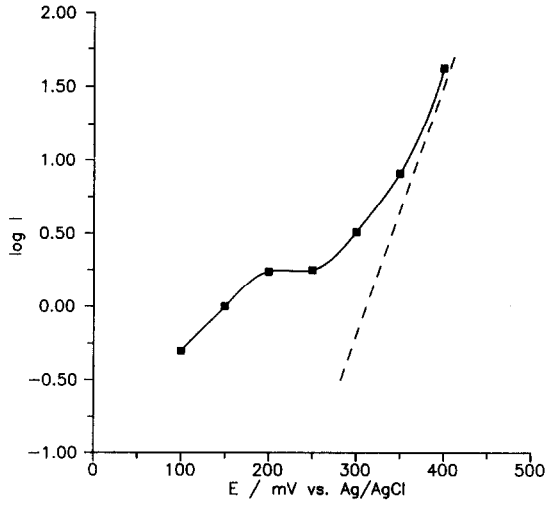

Figure 4
A Tafel plot of 2 mM PAPP in buffer E. An ideal slope of 59 mV decade^{-1} is indicated by the dashed line.

tration than that which can be detected in purity analysis. An alternative, and the most likely explanation, is that PAPP has hydrolysed to *p*-aminophenol to some extent. The oxidation potential of the hump agrees well with the behaviour of *p*-aminophenol shown in Fig. 3. Further support of this view is given by the observation that a peak was observed at the expected position for *p*-aminophenol when the reagent was run through a C_{18} chromatography system. The half-life of PAPP has previously been reported to be 7 days in ammonium carbonate buffer at pH 9.0 [17]. A higher FIA-blank was registered when PAPP was dissolved in glass containers than when plastic containers were used.

The PAPP concentration for the substrate incubation was chosen to be 2 mM, i.e. about 30 times higher than the reported K_M for *p*-aminophenyl phosphate [16]. It should be high enough to ensure a zeroth order reaction of PAPP and as low as possible to reduce the contribution to the current from the PAPP preparation. The peak current at the selected potential, +250 mV, was about 2 nA when 2 mM PAPP was injected.

Product instability

It is well known that many phenols are unstable in alkaline solutions and *p*-aminophenol is no exception. The para-substitution promotes oxidation and *p*-aminophenol is thus oxidized more easily than the phenol itself. This property is advantageous electrochemically but disadvantageous with respect to the sensitivity towards dissolved oxygen and UV-light. For completely deaerated solutions the rate of decomposition of *p*-aminophenol does not increase noticeably from pH 5 to 9 [17]. In practice, however, it is almost impossible to keep the solutions in the microtitre plates free from dissolved oxygen. Figure 5 displays the product decomposition at two pHs under conditions representative for practical handling of samples. It can be seen that the stability is much higher at pH 8 than at pH 9. The former pH was therefore selected although the pH-optimum for alkaline phosphatase with PAPP

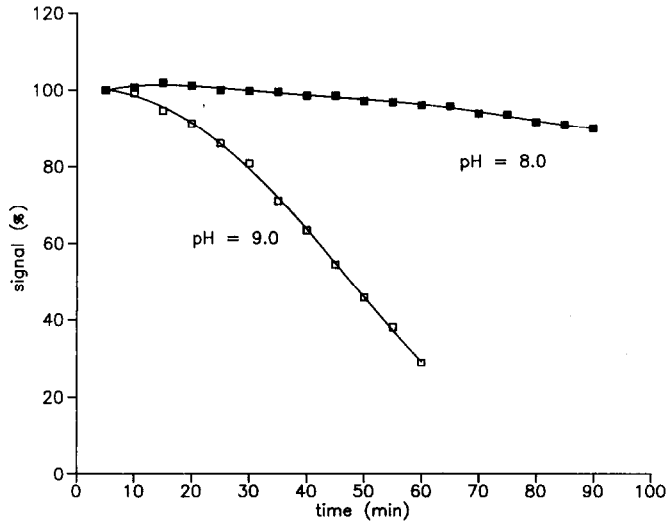

Figure 5
Instability of 1 μM *p*-aminophenol in 0.1 M Tris–HCl at (□) pH 9.0 and (■) pH 8.0. The solutions were deaerated before being added to the microtitre wells. No precautions were taken to prevent the plates from light exposure during the experiment.

as the substrate is pH 9 [17]. A disadvantage with this selection is that the activity of free alkaline phosphatase only is half as high at pH 8 than at the higher pH.

Since the microtitre wells were incubated simultaneously and the FIA determinations of *p*-aminophenol were made sequentially, there had to be a time lag before all the samples were injected and the enzyme activity must therefore be stopped at a fixed time.

Ethanol is an efficient stopping solution at concentrations over 30%. An addition of ethanol also stabilized the product to some extent, probably due to lower solubility of oxygen in the solution. Both the electrochemical response of *p*-aminophenol and the signal-to-noise ratio was decreased, however, and this approach was therefore considered to be less promising. The enzyme activity could also be stopped by lowering the pH to 4.0, but the hydrolysis of PAPP became faster at this pH than at alkaline pH, resulting in a higher FIA-blank. Since the sensitivity of the assay depends on the blank response it should be kept at a minimum.

The best solution seemed to be to stop the enzyme reaction with a phosphate buffer at pH 8.0 and to inject the samples at a high rate (more than one sample per min). However, it was still necessary to make corrections for the instability of PAPP and *p*-aminophenol.

Sensitivity and precision

According to Ekins [4] the theoretical sensitivity of a competitive immunoassay depends on both the affinity constant of the antibody, K_{aff}, and the detection system. The lowest detectable antigen concentration in a well, C is given by

$$C = (\sigma/R)/K_{aff},$$

where σ/R is the relative standard deviation in the measurement of the response, R, and includes both experimental errors and deviations in product detection.

The limit of detection in ELISA will thus depend on the standard deviation of an antigen-free sample, the so called zero-dose response, B_0. There will be at least three factors which affect this quantity, namely the analytical precision of the product determination, the effects of variations of the Non-Specific Binding (NSB) and of the FIA-blank. It was found that NSB did not give any additional contribution to the signal and the normal subtraction of NSB will thus in practice be replaced by a subtraction of the FIA-blank.

The standard deviation of the FIA-blank was 6% after corrections for instability, and the level of response was about 50 times higher than the detection limit for *p*-aminophenol. This was probably due to the presence of *p*-aminophenol in the PAPP reagent as discussed above. The high level of the FIA-blank made it necessary to use a larger amount of conjugate than that which would have been necessary if the limit of detection of *p*-aminophenol had been the only factor to consider. The FIA-blank, which corresponded to about 12% of B_0 was subtracted from both B_0 and the analyte response, B. This subtraction procedure will amplify the relative standard deviation of the B-value.

The time delay in the product determinations made it necessary to correct for the instability of the reagent and the product. Therefore, the time dependencies of the FIA-blank and the zero-dose response were fitted to linear equations, which were used to calculate corrected values of B/B_0.

In the ELISA reported here budesonide could be quantified in the range 3–150 fmoles (see Fig. 6). K_{aff} is 1.63×10^{10} l mol^{-1} for anti-budesonide [13], and σ/R was about 4% in this

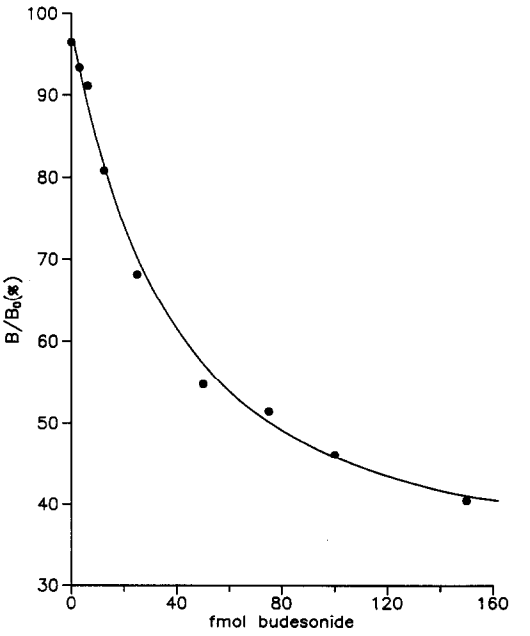

Figure 6
Standard curve for budesonide in pure buffer solutions. Each value is the mean of duplicate determinations.

Table 1
Precision of the ELISA using pure solutions

Femtomole budesonide	B/B_0	RSD (%)*	Found amount budesonide	RSD (%)*
10.0	84.7	3.4	9.7	24.2
30.0	67.7	3.8	29.0	12.7
60.0	56.8	4.3	51.4	13.2
120.0	44.4	8.2	120.8	34.0

*$n = 8$.

work. According to the equation above this results in a C of 2.4 pM, (0.5 fmoles well^{-1}).

The variation of B/B_0 was between 3.4 and 8.2% in the range 10–120 fmoles budesonide per well. This may be due to variations in the amount of adsorbed antibody on the microtitre plate, adsorption to pipette tips and containers, dissociation of the enzyme conjugate and analyte from the antibodies during the washing steps and product instability. The characteristics of the slope of the standard curve resulted in an assay precision of 12.7–34.0% referring to amount of analyte (Table 1).

Plasma samples

Cross-reactivity towards structurally similar substances is of great concern when analysing authentic plasma samples with immunological techniques. In this application cross-reactions may be expected from endogenous steroids or from metabolites of the drug. Structurally different compounds may also interfere if they are present in high concentrations. These interferences become more pronounced as the concentration of analyte is lowered, because of the large ratio between the cross-reacting substances and the analyte.

A solid-phase extraction was found to remove much of the endogenous cross-reacting compounds, and as far as can be seen, the LC-fractionation separated the analyte from the remaining interfering substances. The chromatogram in Fig. 7(a) shows an efficient separation of some important endogenous steroids from budesonide. It has previously been shown that most budesonide metabolites also separate from budesonide in a similar LC-system, since they are generally more hydrophilic [22]. The cross-reactivity for different fractions of blank plasma is shown in Fig. 7(b). It can be seen that the LC-separation removes the majority of the cross-reacting compounds. Another advantage of sample pretreatment is

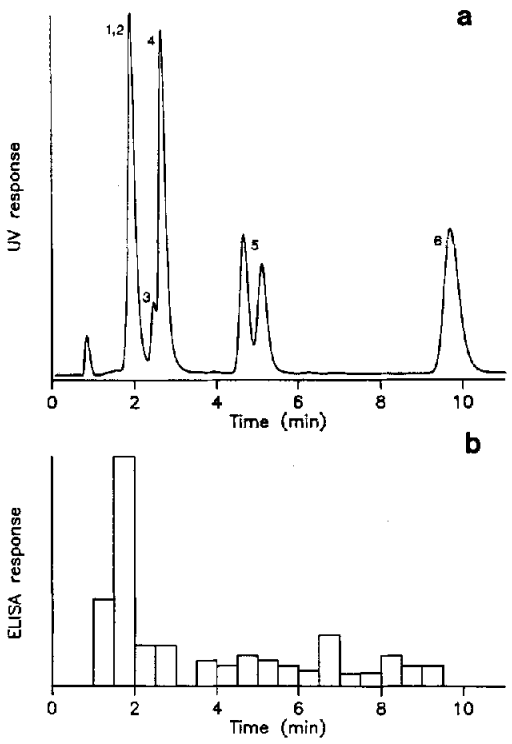

Figure 7
(a) LC-separation of steroid standards. 1.5 nmoles were injected of (1) cortisone, (2) cortisol, (3) corticosterone, (4) 11-deoxycortisol (5) budesonide (*R*- and *S*-epimers) and (6) progesterone on a C_{18}-column, thermostatted to 40°C. The eluent was 35% ethanol in formic acid (5 mM) and the flow rate was 0.5 ml min^{-1}. (b) Cross-reactivity towards anti-budesonide of an LC-fractionated blank plasma sample. The highest value corresponds to 22 fmoles of budesonide.

that a rather large plasma volume can be used to achieve a low limit of quantification.

Analysis of plasma samples, spiked with budesonide, showed that the quantitable concentration range was from 10 to at least 100 pM, with a precision of 27.8–43.4% (see Table 2). The precision for plasma samples was thus worse with than without the pretreatment steps. The reasons are probably decomposition of the analyte or contamination problems during the evaporation steps. The same pre-

Table 2
Precision of the ELISA for pretreated plasma samples

Real conc. (pM)	B/B_0	RSD (%)*	Apparent conc. (pM)	RSD (%)*
9.8	93.7	5.2	12.1	43.4
24.4	78.0	7.9	30.1	27.8
97.8	53.1	11.2	95.3	33.7

*$n = 6$.

cision drop was also observed when evaporating pure solutions of the analyte.

Conclusions

The analytical method reported here has a potential of determining budesonide down to 10 pM in plasma due to a sensitive immunoassay and large plasma volumes. Budesonide has previously been determined quantitatively with LC–radio-immunoassay down to 200 pM [11] and with LC–MS thermospray down to 100 pM [12]. The precision for the LC–RIA and LC–MS methods at the limit of quantitation was 8 and 10–18%, respectively. It has thus been demonstrated that the method presented here can provide an enhanced sensitivity compared to the previously described methods. The precision is not yet sufficient for routine analysis, however.

The pre-separation step for the plasma samples minimizes the risk of interferences from endogenous steroids and budesonide metabolites, but decreases the precision substantially. It is possible to automate the solid-phase extraction with commercially available equipment connected to the LC-fractionation system [23], thus removing one of the critical evaporation steps. Work at picomolar concentrations put special demands on the environment and an automatic handling will reduce sample exposure and increase the precision. The manual injection of *p*-aminophenol into the FIA-system can be replaced by a rack-based auto injector, taking samples directly from the microtitre plate. A possible way to improve the ELISA precision could be by using a procedure in which the wells were coated by excess secondary antibodies or protein G instead of primary antibodies [24].

When the available plasma volumes are much smaller than the 2.5 ml required at present there is a need for even lower quantitation levels than those reported here. Determinations of steroids at even lower concentrations will also demand ever decreasing quantitation levels. It is therefore worthwhile to consider how the limitations of the present method can be reduced. The strategy most closely at hand is to reduce the blank response from the enzyme substrate in the FIA-system but available data on the chemical stability makes it unlikely that there is much to be gained.

The approach reported in this paper can also be applied to many other steroids and to other low molecular weight compounds provided that antibodies with sufficiently high affinity constant can be raised towards them.

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