Development of sampling and bioselective techniques for on-line clinical biosensors
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Chapter 6

Analysis of cortisol with a flow displacement immunoassay

Results! Why, man, I have gotten a lot of results. I know several thousands things that won't work (Thomas E. Edison).

This study investigates a flow immunoassay for cortisol based on displacement of labelled antigen analogues from immobilized antibodies. On a displacement column, two antibodies with different affinities were immobilized on protein-A Sepharose, and a construct of cortisol and horseradish peroxidase (cort-HRP) was associated to the antibodies. Perfusion of the column with cortisol displaced a proportional amount of cort-HRP. On-line detection was performed with an electrochemical detector measuring cort-HRP directly in a flow injection system. For investigating some characteristics of the displacement system, cort-HRP was also detected off-line, with a substrate for HRP, which changes OD_{492}. We tested our system in buffer as well in serum for two days at a flow of 4-6 µl min^{-1}. Analysis of the data show a detectable displacement signal at physiological cortisol concentrations. The specificity, stability of the columns and the influence of the antibody affinity were studied.

Introduction

Total serum cortisol concentrations are 20-200 µg l^{-1} (55-550 nM) of which over 90 % is bound to proteins and has daily fluctuations. To get accurate measurements of the amount of cortisol or of the circadian profile, one needs to measure blood several times a day. Besides this, for some clinical diagnosis, it is necessary to know the exact cortisol fluctuations during the day (Sachar et al. 1973). To comply with this, it is necessary to develop a technique, which enables analysis of continuous samples.

There are several methods to measure cortisol in body fluids. The conventional method, analysis of sera with a HPLC is very labour intensive (Oka et al. 1987). Radio immunoassays have been used for some time now (Huang and Zweig, 1989), but they have the disadvantage of using radioactive compounds.

New techniques, using antibodies and labelled cortisol have been developed during the last decade (Gosling et al. 1993). Nowadays, there are some fully-automated analysers available for the analysis of cortisol, such as Enzymun-Test Assay (Boehringer), ACS:180 (Ciba Corning Diagnostics) (Yatscoff et al. 1996) and Stratus (Baxter) (Rogers et al. 1986). Most of these are based on competitive antibody-antigen assays, where immobilized antibodies are incubated simultaneously with a sample and a known amount of enzyme-labelled cortisol. The cortisol concentration is, therefore, inversely proportional to the enzyme activity. Also, a piezoelectric immunosensor (Attili and Suleiman, 1995) and capillary electrophoresis method (Schmalzing et al. 1995) for cortisol have been studied recently. However, none of these methods allow the continuous monitoring of cortisol in vivo. For this, a homogeneous assay is essential. One suitable technique for on-line analysis is a displacement system, described by Kusterbeck, Rabbany et al., e.g. for cocaine and dinitrophenol (Kusterbeck et al. 1990; Wemhoff et al. 1992; Rabbany et al. 1994; 1995; Yu et al. 1996). Here, antibodies are covalently immobilized to column material or fused silica (Narang et al. 1997b), and saturated with fluorophore or radioactive labelled analyte-analogues. Discontinuous sample analysis is achieved after perfusion of the samples through the column and measuring the released label. Measurements with these columns are performed up to 15 times (Yu et al. 1996). Recently, the displacement
technique has also been described for the off-line analysis of cortisol. Kronkvist et al. (1997) have immobilized antibodies and an alkaline phosphatase labelled cortisol-analogue on a column. Between every measurement the column is regenerated and the flow applied (0.2 ml min$^{-1}$) is not suitable for continuous sampling.

Here, we describe a technique, which can be used for both discontinuous and continuous analysis. We immobilize cortisol antibodies to Protein-A Sepharose and associate horseradish peroxidase labelled cortisol (cort-HRP) to these antibodies. The saturated immobilized antibodies are placed in a column in a buffer flow. When a sample containing cortisol is introduced, a proportional amount of cort-HRP is displaced from the binding sites of the immobilized antibodies and subsequently detected downstream. In principle, the detection of the enzyme is performed with an electrochemical detection of cort-HRP. For this, glucose oxidase, placed downstream of the column, produces hydrogen peroxide, the substrate of the released cort-HRP. The electrochemical detection (ECD) can be applied in the future in small, relatively cheap and low energy consuming devices (McNeil et al. 1997). Moreover, the enzymatic labelling of the antigen with HRP increases the sensitivity. This enables lower perfusion rates of the column, thus enhancing the stability of the system and possible incorporation in body fluid sampling devices such as microdialysis (De Boer et al. 1991) or ultrafiltration (Moscone et al. 1996; Kaptein et al. 1997). Characteristics of the displacement system are studied analysing cort-HRP off-line, measuring the enzyme activity photometrically. We analysed cortisol in different (physiological) concentrations in buffers. The specificity and sensitivity of the displacement reaction is investigated for the antibodies with different affinities by measuring the cort-HRP concentration after perfusions with glucose and cholesterol. We also investigate the system with ultrafiltrated sera.

Materials and methods

Materials

Chemicals and antibodies

Protein-A Sepharose was obtained from Pharmacia, Biotech, Uppsala, Sweden. The Mαcort-H, a high affinity monoclonal antibody (K=1.2 10$^9$ M$^{-1}$) and Mαcort-L, a low affinity monoclonal antibody (K=2.1 10$^7$ M$^{-1}$) against cortisol were obtained from Fitzgerald Industries Internationals Inc., Concord MA (clone M94144); cortisol-HRP (cortisol conjugated at the 3-position to horseradish peroxidase) from Eurogenetics, Tessenderlo, Belgium. Glucose oxidase (EC 1.1.3.4, grade I) was obtained from Boehringer Mannheim, Germany, ureum peroxide tablets from Organon, Weesp, The Netherlands and Kathon CG from Rhom and Haas, Croydon UK. Ferrocenemonocarboxylic acid (FcA), Tween 20 and o-phenylene diamine dihydrochloride (OPD) tablets were obtained from Sigma, St. Louis, MO, whereas other chemicals were of pro-analysis quality and purchased from Merck, Darmstadt, Germany. Sera were obtained from the University Hospital of
Groningen, The Netherlands. They were ultrafiltrated with fibres of dialysis membrane AN69HF (Filtral 16; Hospital Ind., Meyzien, France) as described by Moscone et al. (1996).

**Buffers**

Special buffer (SB) consisted of 0.35 M NaCl, 2.5 mM KCl, 8 mM Na$_2$HPO$_4$, 2.5 mM KH$_2$PO$_4$, 0.03 vol.% Tween-20 and 0.1 vol.% Kathon CG. For the glucose buffer (GB), 5 mM glucose was added to SB. The FcA buffer (the buffer for the flow injection analysis in the detection system) contained 0.5 mM FcA, in GB. The substrate for the off-line quantification of the HRP in the eluent was made as follows: a 4 mg OPD tablet was dissolved in 10 ml distilled quarts water with 100 µl ureum peroxide (in 10 ml). The reaction was stopped with 50 µl 1.5 M H$_2$SO$_4$.

**Equipment**

For the displacement unit, a Minipuls 3 peristaltic pump (Gilson Medical Electronics, WI) was used with clear standard pump tubes (Skalar, Breda, The Netherlands). The columns were made in 25 µl transferpettor-cap (Brand, Germany). The OD$_{492}$ of the sampled fractions was measured with a Lucy1 spectophotometer (Anthos, Salzburg, Austria).

For the flow injection analysis, a HPLC pump (LKB 2150, Pharmacia) was connected to a Marathon HPLC Autosampler (Spark Holland, Emmen, The Netherlands). The sampler was equipped with a Rheodyne 7010 Valve (Cotati, CA) with a loop (homemade, 1.25 µl). Glucose oxidase was immobilized with a 0.01 µm cellulose nitrate filter obtained from Sartorius (Gottingen, Germany). The electrochemical cell was a thin layer-type cell (AMOR, purchased from Spark Holland, Emmen, The Netherlands), with a glassy carbon working electrode held at 0.00 mV relative to an Ag/AgCl reference electrode and a Teflon/carbon counter electrode connected to a potentiostate (Decade, Antec Leyden B.V., Leiden, The Netherlands).

**General description of the displacement system**

Our cortisol analysis system was based on a displacement detection. Antibodies were immobilized on column material, followed by perfusion with cort-HRP to bind to the antigen binding sites (ABS) of the antibodies. The experimental set-up is shown in Fig. 6.1. The column (displacement unit) was perfused continuously with varying amounts of cortisol or other contents. Cort-HRP in the eluent of the column was analysed either off-line or on-line.

In off-line analysis, the continuous sample was divided into 10 minutes-fractions (see Fig. 6.1A) and analysed with a colour substrate. On-line analysis was performed in a flow
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injection analysis system. In this, cort-HRP was detected electrochemically. The displacement unit was coupled to a second flow system with a valve to a connection loop, which enables injections of the samples into a relatively higher flow system. The buffer in this system was the same as used for perfusion of the displacement column but also contained FeA. Immobilized glucose oxidase in this system produced H$_2$O$_2$ from glucose. The HRP reduced the H$_2$O$_2$, oxidizing ferrocene(II) to ferrocene(III). At the electrode ferrocene(III) was then reduced back to ferrocene(II). This set-up enables incorporation of the detection system in an on-line ECD system without an additional fluid system, for in body derived fluids glucose is often present in excess, and may serve as substrate-producing substance.

**Fig. 6.1. Schematic representation of the displacement detection. A. off-line; B. on-line.**

**Preparation and operation of the displacement unit**

Glass wool was placed at the bottom of a part of a 25 µl transferpettor-cap (10-50 µl internal volume). Tubes, fitted in a Gilson Minipuls 3 pump, were connected to the bottom of the cap. The cap was filled with Protein-A Sepharose. The standard flow of the displacement unit for loading and running was 4-6 µl min$^{-1}$.

100 µl (330 ng µl$^{-1}$ in GB) Mozct-H antibody was immobilized on the protein-A. After this, the column was washed with at least two column volumes, followed by an incubation with 50 µl (64 µg ml$^{-1}$) cort-HRP in GB. A tube at the top of the column
connected the column to the pump and the system was washed for at least 16 hours with GB. The column in the displacement unit was continuously perfused maximally two days with GB or with various concentrations of cortisol. The column was also perfused with SB and 50 µM cholesterol in SB (as a compound present in body fluids with similar chemical properties), or with 5 mM glucose in SB (as a compound with physiologically high concentrations) to determine the specificity of the displacement. Non-protein bound cortisol in filtrated serum was also analysed. Extra cortisol (275 nM) in GB was added before and after filtration.

Description of the off-line detection system

From the displacement unit described previously, the 10 min fractions of the on-line sample were analysed by adding 100 µl substrate to 5 µl of these fractions (see Fig. 6.1). The reaction was stopped with H$_2$SO$_4$ when OD$_{492,max}$ = 1.

Description of the on-line detection unit

As described in above, the on-line detection was performed as a flow injection analysis. Injections of the perfused fluid into the detection unit was performed every 2 min. The flow of the displacement unit was 6 µl min$^{-1}$, the flow in the detection unit was 100 µl min$^{-1}$.

Presentation of the results

Several examples of our experiments are given to illustrate the significance of the various parameters studied. In other cases linear regression between data was calculated. In several experiments the efficiency of the displacement was expressed as the percentage of the ration of the cort-HRP (in mol) eluted and the cortisol perfused (in mol) analogue to the definition of Rabbany et al. (1995).

Results and discussion

Analysis with the off-line detection unit

Specificity of the displacement

The specificity of the displacement reaction was tested in two ways. Firstly, physiological concentrations of cholesterol and glucose were perfused; these did not displace cort-HRP (Fig. 6.2A). Secondly, ultrafiltrates of sera (with or without extra cortisol) were tested, showing no more increase of cort-HRP in the eluates than was
expected on the unbound cortisol concentrations in the sera (see Fig. 6.2B). In the serum ultrafiltrates, little cortisol was present because only free cortisol (approximately 0.5 µg l⁻¹) passed the membrane. When 10 µg l⁻¹ cortisol was added to the ultrafiltrate the displacement was similar to the displacement after perfusion with 10 µg l⁻¹ cortisol (about 1.9 10⁻¹⁴ mol) in buffer. In some serum filtrates samples unknown compounds disturbed the HRP-activity. In general, these were not present or physically separated from the displaced cort-HRP (see Fig. 6.2B).

Fig. 6.2. Cort-HRP release and activity in fractions after perfusion. A. Specificity of the displacement. Bars indicate the cort-HRP activity present in the fraction. Arrows indicate the perfusion starts: 275 nM cortisol; 5 mM glucose; 50 µM cholesterol. B. Displacement and enzyme activity after serum perfusions. Bars: Column loaded with cort-HRP-saturated antibodies; Line: Unloaded column perfusate analysed with extra cort-HRP (OD devided by 3); Perfusions with samples were performed for 20 minutes as indicated. 1. 275 nM cortisol buffer; 2. dialysed sera and buffer; 3. sera and 275 nM cortisol buffer, dialysed; 4. dialysed sera and 275 nM cortisol buffer.
Fig. 6.3. Displacement experiments. Off-line detection of cort-HRP in 10 minute fractions from continuously perfused columns. Arrows indicate the perfusion starts of 20-min cortisol perfusion in the concentrations indicated (nM). A. column with \( \text{M}^c \text{ort-H} \) one day after column preparation; B. column with \( \text{M}^c \text{ort-L} \) one day after column preparation; C. column with \( \text{M}^c \text{ort-H} \) four days after column preparation; D. column with \( \text{M}^c \text{ort-L} \) two days after column preparation.

Influence of the antibody affinity on the signal

Fig. 6.3 shows the elution profiles of two columns loaded with high affinity or low affinity antibodies. The first cortisol perfusion of the column with \( \text{M}^c \text{ort-H} \) showed a lower displacement signal as perfusion of the same amount of cortisol later that day.

The ratio of the amount of ABS and of cort-HRP bound to this and of the amount of cortisol on the column when 275 nM cortisol is perfused 20 minutes equals to \( \text{ABS}:\text{cort-HRP}:\text{cortisol}=12:3:1 \). Thus, the ABS were not saturated with cort-HRP when the displacement experiments started. This might explain why the first cortisol perfusion did not displace cort-HRP as much as later cortisol perfusions, presumably because the first
cortisol became partially trapped at free antibody binding sites. However, perfusion for 20 minutes at a concentration of 275 nM cortisol only added 3.3 $10^{-11}$ mol cortisol to the column and did not theoretically fill all the free sites, suggesting that some ABS were not available for antigen binding. Wemhoff et al. reported a lower release at the first injection of antigen even after loading the antibodies with a 100-fold excess of antigen homologues (Wemhoff et al. 1992). They explained this by assuming that there are empty ABS after the preparation of the column. We hypothesize that some ABS cannot bind to relatively large cort-HRP due to steric hindrance, but are still accessible for the small unconjugated cortisol.

The initial decrease of displacement in the first cortisol sample for the Mαcort-H column was not seen after the first day, although the absolute signal decreased. There are several explanations for this. One is that the amount of cort-HRP on the columns decreases as a result of the perfusion. However, the spontaneous release of cort-HRP was less than $1 \times 10^{-16}$ mol min$^{-1}$. The release of cort-HRP caused by displacement from 275 nM cortisol was at most $5 \times 10^{-16}$ mol min$^{-1}$ (calculated from 10 min fractions). Compared to the amount of cort-HRP present on the column ($8 \times 10^{-10}$ mol), the absolute amount of cort-HRP displaced was not changed significantly. Another explanation for the decrease of signal is that the apparent binding constants differ for the antibodies. Wemhoff et al. (1992) have described a similar phenomenon. It can be explained by the steric hindrance described previously, but it may also be (partly) an effect of inhomogeneity of the cort-HRP. For example, the number of cortisol molecules per HRP molecule may influence the affinity as described for a similar process: the binding of antibodies (with two ABS) compared to that of the Fab fragment of the antibody (with one ABS) (Nygren et al. 1987). A last explanation is that the cort-HRP enzyme activity decreases during the perfusion period. In this case, the calculated displacement rates will be lower than experimentally found.

The Mαcort-L column did not have a lower displacement for the first injection of cortisol, but here the displacement on the first day was not dependent on the concentration of cortisol but only on how long after the preparation of the column the cortisol was perfused. However, the signal seemed to be more concentration dependent on the second day than on the first. The reason for this is unknown, although it might be explained by assuming that there are more unstable complexes on the first day, for example because of steric hindrance for the large cort-HRP onto the column material. These can be easier released in their dissociation phase in the presence of cortisol. On the second day, these unstable complexes might have dissociated already.

For analysis of lower concentrations of cortisol-HRP, the antibody with the low affinity was preferred. The displacement from the high affinity antibody was too small to distinguish from the spontaneous dissociation, whereas the column with Mαcort-L showed concentration dependent signals (data not shown).
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Quantification of the signal

Repeated perfusions of $3.3 \times 10^{-11}$ mol cortisol in 20 minutes (275 nM) showed a decrease from 0.05% to 0.02% of this displacement efficiency for on the second day for the columns with $\alpha$-cort-H. Measurements on the same day did not show significant differences (see Fig. 6.4). The displacement efficiency for $\alpha$-cort-L was not calculated, for this was highly influenced by the perfusion time, i.e. the amount of cort-HRP dissociated from the column.

![Graph](image)

**Fig. 6.4.** Displacement rate ± SEM (percentage release of cort-HRP/cortisol) for cortisol perfusion of $3.3 \times 10^{-11}$ mol.

Using $\alpha$-cort-H, the displacement rate was not significantly different at the applied concentrations of cortisol used for perfusion. The cort-HRP present in the eluate showed, therefore, a linear relation ($r=0.99; p=0.006$) with the perfused cortisol concentration (Fig. 6.5). The intercept of the y-axis showed the spontaneous dissociation (0.16 fmol per sample of 10 minutes).

![Graph](image)

**Fig. 6.5.** Amount of cort-HRP in fraction for perfusion with different cortisol concentrations.

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Analysis of cortisol with a flow displacement immunoassay

Analysis with the on-line detection unit

Fig. 6.6 shows a recording of in vitro experiments of our FIIA system. Above the baseline, where no column fractions were injected gave the displacement column eluates a basal background signal, even when no cortisol was perfused (the spontaneous dissociation). Upon perfusion with cortisol, this signal increased. We were able to measure physiological cortisol concentrations every two minutes. Cholesterol did not enhance the signal (data not shown).

![Graph showing cortisol concentration over time](image)

Fig. 6.6. On-line FIIA detection of cort-HRP at different physiological cortisol concentrations. Shown is a profile in which buffer is run continuously, with a 10-min 77 nM cortisol and a 15-min 39 nM cortisol perfusion.

Conclusion

This study demonstrates the potential of the displacement technique. Here, we applied this approach for continuous and batch-wise analysis of cortisol. Others (see Kusterbeck et al. 1990; Wemhoff et al. 1992; Whelan et al. 1993; Rabbany et al. 1995; Yu et al. 1996) have also described batch-wise displacement analysis, although our method reveals some differences. Firstly, we use another analyte in another sample matrix; secondly we perfuse at a much lower flow (6 µl min\(^{-1}\) instead of one ml min\(^{-1}\)); thirdly, we use another (indirect) label and detection system. And most important, we perfuse the displacement column continuously with on-line samples. Our results show a 100-fold lower displacement efficiency than Rabbany et al. (1994; 1995). This may have to be attributed to the antibody, having a high equilibrium constant of \(1.2 \times 10^9\) M\(^{-1}\), resulting in a low displacement efficiency. The unoccupied binding sites, catching cortisol in the sample, do not offer an explanation (except for the first injection), because then the release of cort-HRP would have been increased during the experiment. When the detection of the label is sensitive
enough, a high affinity antibody with a low displacement efficiency and low spontaneous
dissociation and perfusion with a low flow rate might be preferable, because of the good
stability and the longer lifetime of the column. This is clearly demonstrated with the
Mαcort-L, where the sensitivity is largely reduced during extensive usage. Especially using
high flow rates, the displacement efficiency is high, but the sensitivity decreases faster.
However, low affinity antibodies are preferable for the detection of low concentrations of
the analyte, because of the lower detection limit. Further research has to show whether this
principle is more general, or whether this is only valid for the antibodies used here.

With the off-line detection of cort-HRP, it is possible to study many parameters
simultaneously. Multichannel pumps enable serial running of columns, using simple
apparatus (a pump and an ELISA reader) only. Because of its simplicity, the off-line
analysis is a powerful research tool for further development of the on-line system. The
on-line system itself provides a useful tool in research as well as in clinical applications,
because of its minimal labour requirement and possibility of automation (Hitzmann et al.
1995).

In on-line body fluid analysis, interference of other compounds on the detection
systems might lead to false measurements, as sometimes is seen in the detection of HRP.
Therefore, the labelling method has to be chosen with great care.

In summary, the present report shows a novel application of the immunological
technologies in the development for the fast and specific detection of a wide variety of
analytes for biomedical research. The principle of this approach is to displace enzyme
labelled antigens from an antibody complex by an analyte in a flow system, allowing
sensitive electrochemical detection. We characterize some properties of the system,
especially the difference between the displacement from antibodies with a high or low
affinity.

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Addendum chapter 6

After publishing the above chapter of “analysis of cortisol with a flow displacement immunoassay”, there have been performed some experiments, which results are worth mentioning here.

Firstly, we tested the displacement in a continuous flow system with electrochemical detection. The general set-up was comparable to the flow injection system as described, with some changes. The first is that there are no pulses, but a continuous perfusion with cortisol-containing buffer. The second is the size of the column (smaller: 1.5 µl) and flow rate (3 µl min\(^{-1}\)). With this, we were able to detect 1.25 µl of 22 µg l\(^{-1}\) cortisol. The peak width is 10 min at half height and shows some tailing, but this is caused by the size of the column and the flow used. For analysing continuous samples, the peak width may become a problem. The continuous samples enable us to monitor the concentration of the cortisol during the day, and will give valuable data. Miniaturizing the column as well as the electrochemical detection unit will increases the time resolution at low flow and result in a portable device.

Secondly, we investigated the influence of the ultrafiltrate on the electrochemical detection. As described before, the results of the experiments with sera show inhibition in off-line (coloured-product-based measurements) of the cort-HRP enzyme activity by serum components. Recent studies with fresh sera showed that this interference was due to conservatives or drugs in the sample used in this report. However, in the electrochemical detection systems, there is also an electrochemical disturbance of signal in the cort-HRP displacement fractions. To overcome this problem, an extra size selecting column in the detection unit, separating serum compounds (due to the dialysis step smaller than 20,000 Da) and cort-HRP (42,000 Da) might solve this problem. However, this will make it impossible to create a continuous signal. Therefore, a solution should be found on the detection level itself, either by the application of special (coated) electrodes, or by finding another suitable detection system.

An interesting phenomenon has been observed in the off-line analysis system when cortisol concentrations were decreased further. As in “high” concentrations (around 10 µg l\(^{-1}\)), the antibody with the high affinity for cortisol gives the best, quantitative reactions, for lower concentrations (around 0.05 µg l\(^{-1}\)), the low affine antibody has to be preferred. The displacement from the high affinity antibody is too little to distinguish from the spontaneous dissociation, whereas the column with M\(\alpha\)cort-L shows cortisol-concentration dependent signals.