J. Steroid Biochem. Molec. Biol. Vol. 43, No. 7, pp. 683–692, 1992
Printed in Great Britain. 

0960-0760/92 $5.00 + 0.00


# SYNTHESIS OF A CORTISOL–BIOTIN CONJUGATE AND EVALUATION AS A TRACER IN AN IMMUNOASSAY FOR SALIVARY CORTISOL MEASUREMENT

R. A. DRESSENDÖRFER,[1] C. KIRSCHBAUM,[2] W. ROHDE,[3] F. STAHL[3] and C. J. STRASBURGER[1]*

[1]Medizinische Klinik, Klinikum Innenstadt der Ludwig-Maximilians-Universität, Ziemssenstraße 1, 8000 München 2, [2]Fachbereich I Psychologie, Universität Trier, 5500 Trier-Tarforst and [3]Institut für Experimentelle Endokrinologie, Bereich Medizin (Charité) der Humboldt-Universität zu Berlin, Schumannstraße 20/21, 1040 Berlin, Germany

*(Received 2 December 1991)*

**Summary**—Cortisol 3-(*o*-carboxymethyl)oxime (C3-CMO) and a commercially available biotin-hydrazide derivative were used to synthesize a C3-CMO–biotin conjugate. C3-CMO was converted into a *N*-hydroxysuccinimide ester derivative which in a second reaction step was allowed to interact with the hydrazide derivative of biotin. This simple-to-perform synthesis yielded a conjugate suitable for use as a tracer in immunoassays for cortisol measurement. Employing biotin as the primary probe in a competitive solid phase immunoassay allows for variable end point determination by means of commercially available labeled avidin or streptavidin derivatives. Streptavidin–Europium was used in conjunction with the DELFIA-system for time-resolved fluorometric end point measurement (TR-FIA) throughout the study. In addition, colorimetric end point determination (ELISA) using streptavidin-alkaline phosphatase as a secondary probe was established and evaluated. Both forms of this non-isotopic assay showed excellent correlation with a commercially available radioimmunoassay adapted for salivary cortisol measurement. The lower detection limit was 0.43 nM for a 50 $\mu$l salivary sample. The intra-assay coefficient of variation was 6.7, 4.7 and 4.0% at cortisol concentrations of 2.2, 5.5 and 13.2 nM, respectively ($n = 37$), and the corresponding inter-assay coefficients of variation were 9.0, 8.6 and 7.1% ($n = 50$). The competitive immunoassay requires 1.5 h incubation time and shows robust and reproducible performance. The C3-CMO–biotin conjugate allows for sensitive and flexible end point determination of salivary cortisol levels in immunoassays.

## INTRODUCTION

Cortisol measurements in biological samples by immunoassays have traditionally been made using $^3$H- or $^{125}$I-labeled cortisol derivatives as tracers [1]. The use of scintillation liquid for $^3$H-labeled immunoassays and the half life of $^{125}$I-tracers as well as general environmental and health considerations have made the use of non-isotopic immunoassay labels desirable. In the first generation of non-isotopic cortisol immunoassays the steroid was conjugated to enzymes such as horseradish peroxidase [2]. In these immunoassays a competition reaction takes place between the standard or a sample cortisol ($M_W$: 362.5 Da) and the enzyme conjugate ($M_W$: 45,000 Da). Salivary cortisol, which reflects the non-protein bound fraction of the total plasma cortisol in circulation [3] has been measured after a combined extraction and concentration step [4] or directly after the advent of sensitive direct cortisol radioimmunoassays [5]. The determination of salivary cortisol is of increasing interest in clinical diagnostics [3, 6, 7] as well as in behavioral studies and stress research due to the non-invasive sampling procedure [8, 9].

Since the late 70s the exceptional affinity ($K_A = 10^{15}$ M$^{-1}$) of the biotin–avidin system has been employed in immunoassay systems [10]. After *N*-hydroxysuccinimide ester derivatives of biotin became commercially available, these were easily coupled to $\varepsilon$-amino groups of proteins such as antibodies or peptide-hormones involved in the immunoassay reaction. The end point was determined colorimetrically [11] or by chemiluminescence [12] after an additional incubation step with enzyme- or chemiluminescent-labeled avidin or streptavidin. Biotinylated tracers, in contrast to $^{125}$I-labeled tracers, offer the advantage of virtually unlimited stability and their "specific activity" is not affected by

*To whom correspondence should be addressed.

storage time. To the best of the authors' knowledge there are only a few reports in the literature on direct steroid–biotin conjugates [13–15]. In order to synthesize a low molecular weight cortisol–biotin conjugate for use as a tracer in cortisol immunoassays, we have conducted experiments to couple commercially available derivatives of cortisol and biotin by a simple, new approach.

## EXPERIMENTAL

### *Synthesis of the cortisol 3-(o-carboxymethyl)-oxime (C3-CMO)–biotin conjugate*

In the first reaction step 50 $\mu$mol (21.8 mg) of hydrocortisone 3-CMO (Sigma, Deisenhofen, Germany, No. H-6635) was dissolved in 200 $\mu$l of dry, amine-free *N,N'*-dimethylformamide (DMF) (Merck, Darmstadt, Germany, No.

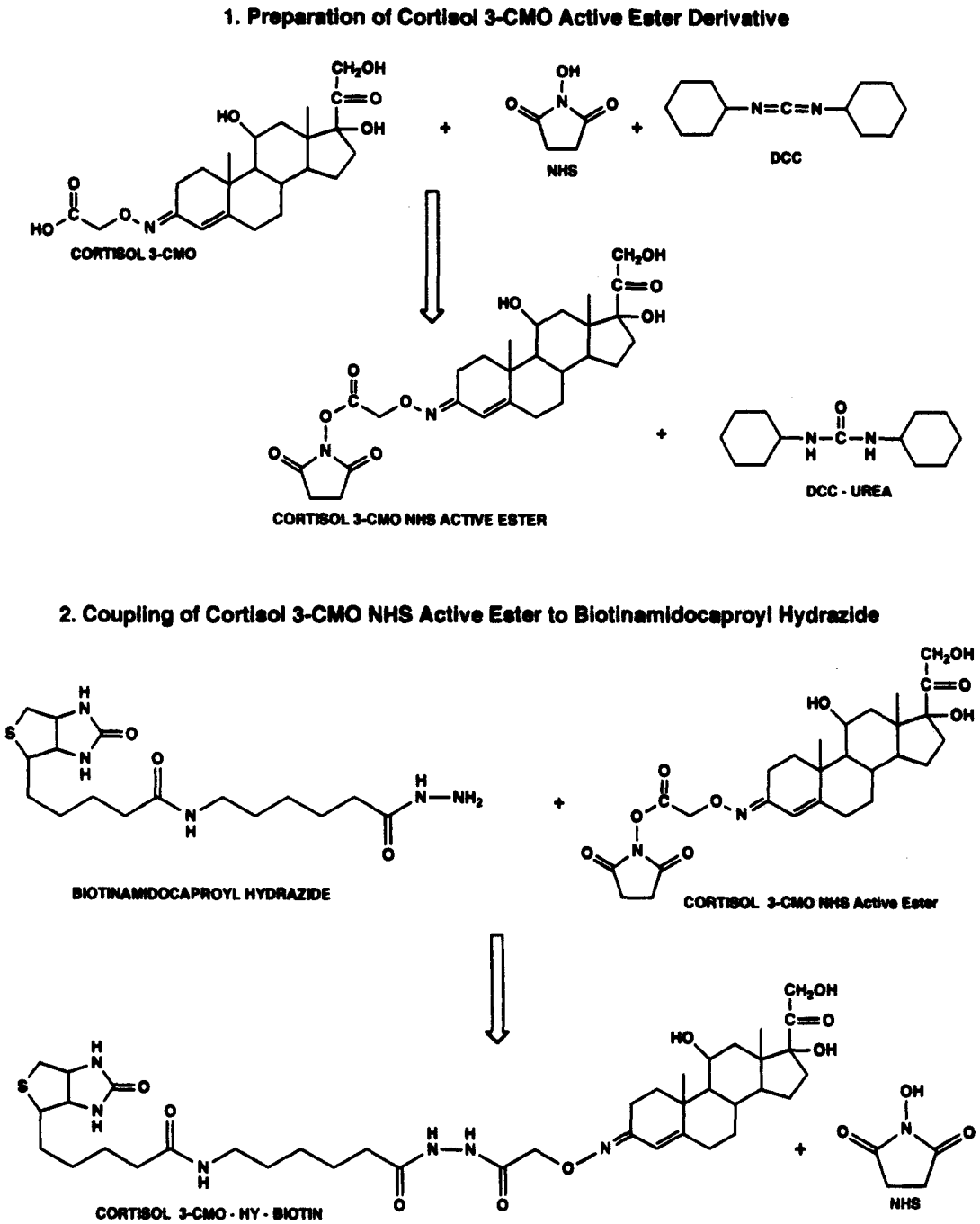


Fig. 1. Pathway of synthesis and proposed chemical structure of the C3-CMO–biotin tracer conjugate.

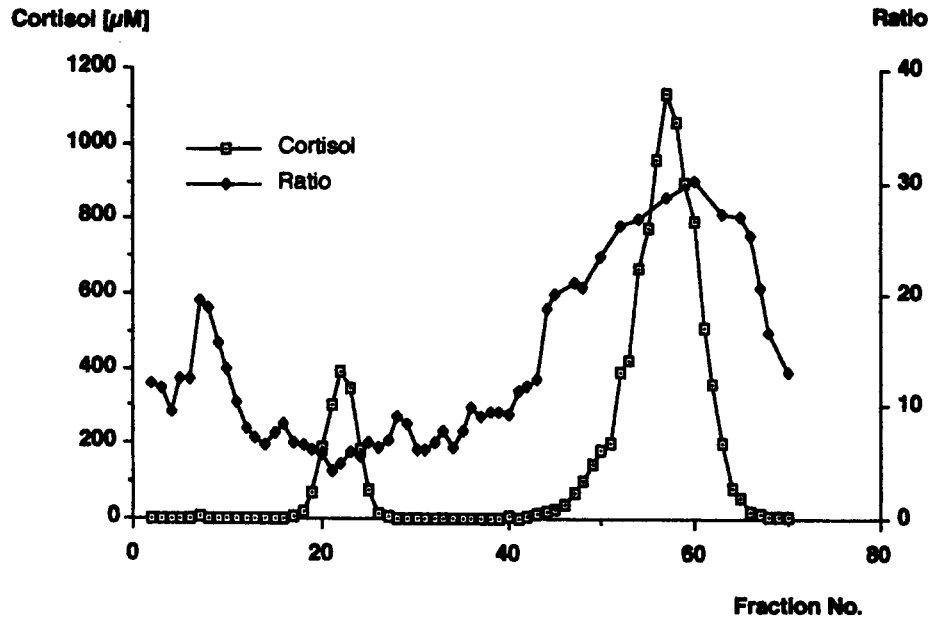


Fig. 2. The synthesized C3-CMO–biotin tracer conjugate was purified by size exclusion chromatography on a Trisacryl GF05 M (10 × 400 mm) column and fractions of 1 ml were collected. The cortisol content was analyzed by RIA and two well separated peaks were found. The signal-to-noise ratio was determined by TR-FIA as described in the Results section. The highest signal-to-noise ratio corresponded to the second cortisol peak.

3053). Equimolar amounts (5.6 mg) of *N*-hydroxysuccinimide (NHS) (Sigma, No. H-7377) and (10.3 mg) of *N,N'O*-dicyclohexylcarbodiimide (Sigma, No. D-3128) were dissolved in 40 and 60 μl of DMF, respectively, and added to the C3-CMO. The reaction mixture was stored at room temperature under light-exclusion for 24 h: the C3-CMO was converted into its NHS ester, and crystal needles of dicyclohexylurea appeared in the mixture. For coupling of the cortisol–NHS ester to biotin, 50 μmol (18.6 mg) of biotinamidocaproyl hydrazide (Sigma, No. B-3770) was dissolved in 200 μl of dimethylsulfoxide (DMSO) (Merck, No. 2952). The completely dissolved biotin derivative was added to the reaction mixture which was left at room temperature for another 24 h under light-exclusion. The proposed mechanism of reaction and structure of final product are shown in Fig. 1.

*Purification of the C3-CMO–biotin conjugate*

Aliquots of this reaction mixture were applied to a 10 × 400 mm Trisacryl GF05 M (Serva, Heidelberg, Germany, No. 67010) column after 1:10 (v/v) dilution in column buffer (50 mM Tris–HCl, pH 7.8). The elution pattern of the column showed two well-separated peaks (see Fig. 2). The cortisol content of the fractions was analyzed by a commercially available radioimmunoassay (MAGIC Cortisol, Ciba Corning Diagnostics GmbH, Gießen, Germany). Cortisol concentrations in the column fractions corresponded to the absorbance at 254 nm.

*Cortisol antiserum*

Cortisol antiserum was raised by repetitive immunization of rabbits with C3-CMO:bovine serum albumin (BSA) as the immunogen. Specificity of the antiserum was determined by addition of increasing amounts of crossreacting steroid as the unknown sample in the immunoassay described in this article. The doses of cortisol and crossreacting steroid hormone effectively displacing 50% of the tracer from the antibody (ED-50) were compared and the respective concentrations were expressed as ED-50 (cortisol):ED-50 (crossreacting steroid) × 100[%].

*Immunoassay design*

Affinity purified swine anti-rabbit immunoglobulin (Dako, Hamburg, Germany, No. Z400) was immobilized onto Maxisorp-microtiterplates (Nunc, Roskilde, Denmark, No. 4-42404) at 100 ng per well in 50 mM sodium-phosphate, pH 9.6. These plates were stored in the coating buffer for up to 3 months without

loss of activity. Cortisol standards were prepared by diluting an ethanolic stock solution of cortisol (10 μg/ml) in a buffer salt mixture prepared in accordance with the German Industry Norm for artificial saliva (DIN 53 160) [16]. Prior to the immunoassay procedure, plates coated with swine anti-rabbit IgG were washed three times in an automatic microtiterplate washer (DELFIA platewash, Pharmacia, Uppsala, Sweden, No. 1296-024), and 50 μl of standard or sample were pipetted to the bottom of the microtiterplate-wells. Subsequently, 50 μl of the cortisol–biotin conjugate (20 pg/well) was added, followed by 100 μl of rabbit anti-C3-CMO antiserum diluted 1:50,000 in assay buffer [50 mM Tris–HCl, 150 mM NaCl, 0.5% (w/v) BSA (Sigma, No. A-7906), 0.05% (w/v) bovine γ-globulin (Sigma, No. G-7516), 0.02% (w/v) Tween 40, 0.02% (w/v) $NaN_3$, pH 7.7]. Incubation was allowed to proceed for 1 h at ambient temperature on a horizontal microtiterplate shaker (DELFIA Plateshake, Pharmacia, No. 1296-001).

*End point determination*

After 1 h of incubation between analyte, tracer conjugate and antibody as well as the solid phase coated anti-rabbit IgG-antibody the incubation was terminated by washing three times. For time-resolved fluorometric measurement (TR-FIA) 20 ng of streptavidin–Europium (Stav–Eu) conjugate (Pharmacia, No. 1244-360) was added to each well in 200 μl assay buffer. The reaction was allowed to proceed for 30 min on a horizontal shaker. After a 6-fold wash step, "enhancement solution" (Pharmacia, No. 1244-105) was added, by which the Europium coupled to streptavidin is transferred into a highly fluorescent complex [17]. TR-FIA measurement was carried out in a 1232 DELFIA Fluorometer (Pharmacia, No. 1232-002). For colorimetric end point measurement, after the immunoreaction and a 3-fold wash step, streptavidin–alkaline phosphatase conjugate (Boehringer, Mannheim, Germany, No. 1089 161) diluted in assay buffer in a concentration corresponding to 20 ng of streptavidin per well was added. After 30 min of incubation the plate was washed six times and 200 μl (10 mM) of *p*-nitrophenyl phosphate (pNPP) (Merck, No. 6850) was added in 1 M diethanolamine buffer, pH 9.8. Substrate development was allowed to proceed at ambient temperature for 45 min, an o.d. reading was carried out at 405 nm.

*Reproducibility*

The intra-assay coefficients of variation were determined by a 37-fold measurement of pooled saliva samples with cortisol concentrations of 2.2, 5.5 and 13.2 nM. The same saliva pools were determined in 50 consecutive assays for calculating the inter-assay variation.

*Investigation of potential interference from serum contamination in salivary samples*

Two saliva pools A and B with cortisol concentrations of 5.52 and 47.7 nM, respectively, were mixed with a serum obtained at 9 a.m. (cortisol concentration 516 nM) in ratios yielding final serum concentrations of 0.05–2.0% of the total volume. In addition, a dexamethasone suppressed serum (cortisol content 36.4 nM) was mixed in the same ratios with saliva pool B. Expected values and measured values were compared.

*Reference-radioimmunoassay*

The commercially available "MAGIC Cortisol RIA" was used in the version adapted for cortisol measurement in saliva as published elsewhere [18]. This immunoassay kit uses [$^{125}$I]histamine-3-CMO-cortisol as a tracer, the antiserum in this kit was raised against C3-CMO–BSA.

*Saliva sampling*

Salivette saliva sampling devices (Sarstedt, Nümbrecht, Germany, No. 51.1534) were used consisting of a 40 × 9 mm cellulose tampon, an inner and outer tube. Ten minutes after rinsing the mouth with water, the cellulose tampon is kept in the mouth or chewed on for 1–3 min. Thereafter, the tampon is transferred to the inner of the two polystyrene tubes and the sample is frozen. Prior to analysis the sample is thawed and centrifuged so that the salivary liquid is transferred to the outer tube. Clear saliva can be pipetted from the outer polystyrene tube after discarding inner tube and tampon. Saliva samples, which by inspection can be diagnosed as contaminated with blood are excluded from analysis.

## RESULTS

*Synthesis and purification of the C3-CMO–biotin conjugate*

The conjugate was diluted as described in the Experimental section and chromatographed

over a Trisacryl GF05 M column. u.v.-Monitoring at 254 nm revealed two major peaks, one around fraction 22, another around fraction 57. The cortisol content of the fractions was determined using the commercial RIA [18]. Fractions of high cortisol concentration coincided with the absorbance peaks at $o.d._{254}$. In addition, the fractions were tested as tracer for maximal binding and displacement by a 2.76 $\mu$M cortisol standard. As outlined in the assay protocol above, 50 $\mu$l of conjugate-fraction diluted to contain 20 pg per well of cortisol-equivalent were pipetted into the well as tracer along with 50 $\mu$l of zero standard or of 2.76 $\mu$M standard. Determinations were performed in duplicate, and the ratio between the signal obtained at maximal binding and the displacement by the 2.76 $\mu$M standard was calculated. The results are presented in Fig. 2, with the second peak showing the highest binding ratio. This peak apparently contains the cortisol–biotin conjugate, while the first peak contains mainly uncoupled cortisol.

*Assay procedure*

The influence of incubation time on the signal obtained and on the discrimination capability of the immunoassay was investigated by varying the incubation time of the tracer, standard or sample, as well as of anti-cortisol antibody in the anti-rabbit IgG coated plates between 0.5 and 4 h. With increasing incubation time, there is a progressive increase of the signal obtained, as well as a slight increase in the concentration required to displace 50% of the tracer from the antibody. After 1 h of incubation the ED-50 is at 7.9 nM cortisol concentration and, after 4 h the ED-50 is 9.2 nM. One hour incubation time was chosen as the compromise between signal intensity and sensitivity mainly for practical

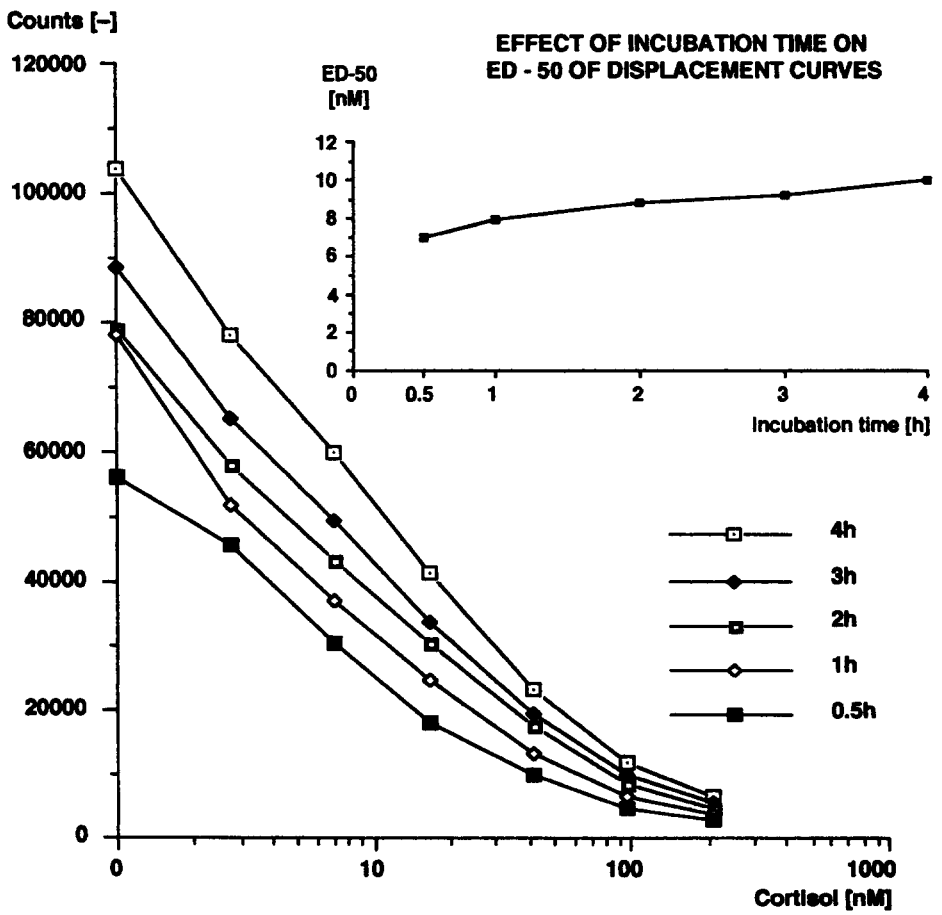


Fig. 3. In the salivary cortisol TR-FIA, the influence of the incubation time on the fluorescence signal obtained and on the ED-50 of the displacement curves was investigated. At longer incubation times, there is a progressive increase of the signal and a slight loss of sensitivity, 1 h of incubation time was chosen as the compromise for practical reasons and assay performance speed.

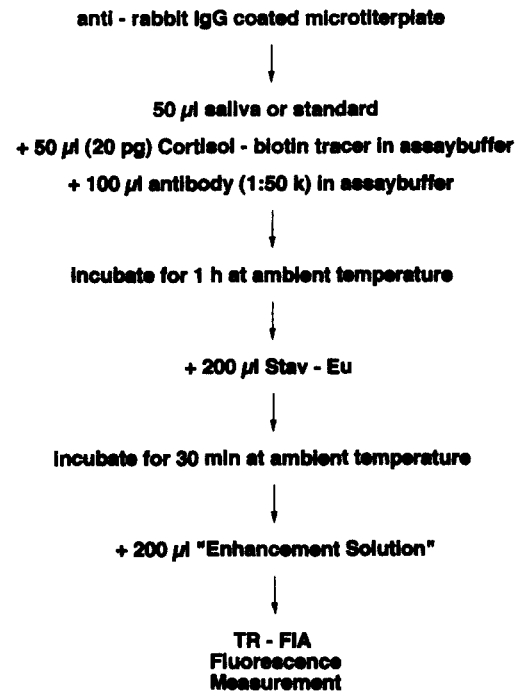


Fig. 4. Assay flow diagram of the cortisol–biotin TR-FIA.

reasons of assay performance speed. The results of the experiment described are shown in Fig. 3, and the final assay procedure in Fig. 4.

Table 1. Specifity of the rabbit anti-C3-CMO antibody as determined by TR-FIA

| Crossreactivity with | (%) |
|---|---|
| Corticosterone | 0.98 |
| 17α-Hydroxyprogesterone | 0.14 |
| 21-Desoxycortisol | 0.36 |
| Aldosterone | 0.014 |
| Cortisone | 11.2 |
| Tetrahydrocortisol | 0.068 |
| Testosterone | 0.003 |
| Prednisolone | 50.6 |
| 11-Desoxycortisol | 33.6 |
| Progesterone | <0.001 |
| Dexamethasone | 0.0013 |

*Specificity*

The displacing potency of potentially crossreacting steroids was investigated by addition of increasing doses of crossreacting steroid as the unknown sample in the immunoassay procedure, and comparison of ED-50 concentrations. In this assay, as shown in Table 1, corticosterone has a relative displacing potency of 0.98%, 17-α-hydroxyprogesterone 0.14%, 21-desoxycortisol 0.36%, cortisone 11.2% and prednisolone 50.6%. Dexamethasone crossreactivity was low (0.0013%).

*Sensitivity*

The sensitivity of the TR-FIA method was determined by 30-fold determination of zero

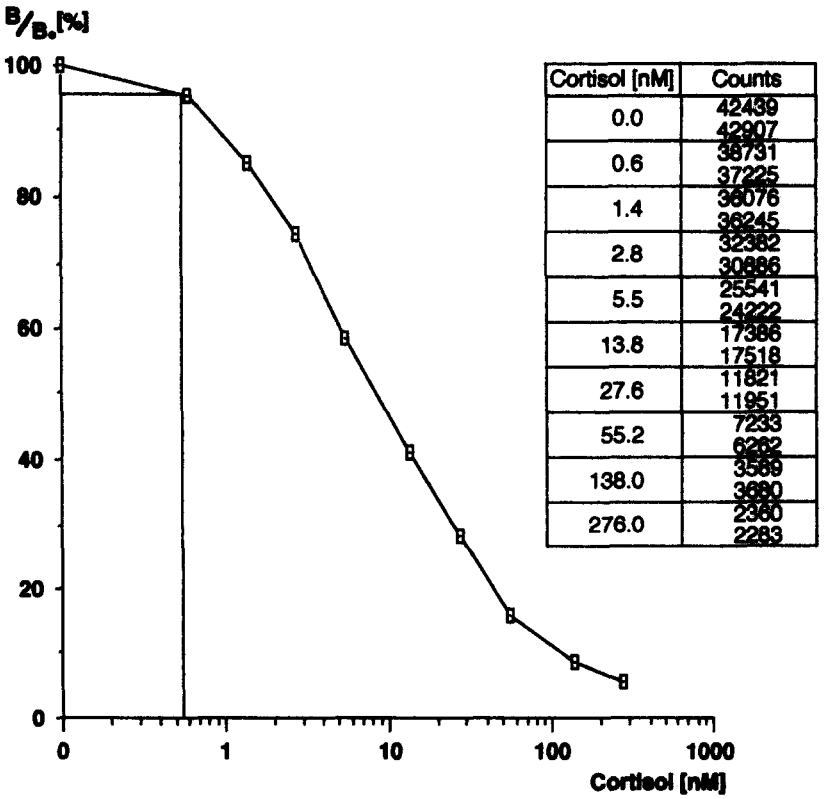


Fig. 5. Displacement curve and data of the cortisol–biotin TR-FIA (50 µl sample). The lower detection limit of 0.43 nM (mean −2 SD) was determined by 30-fold determination of zero binding.

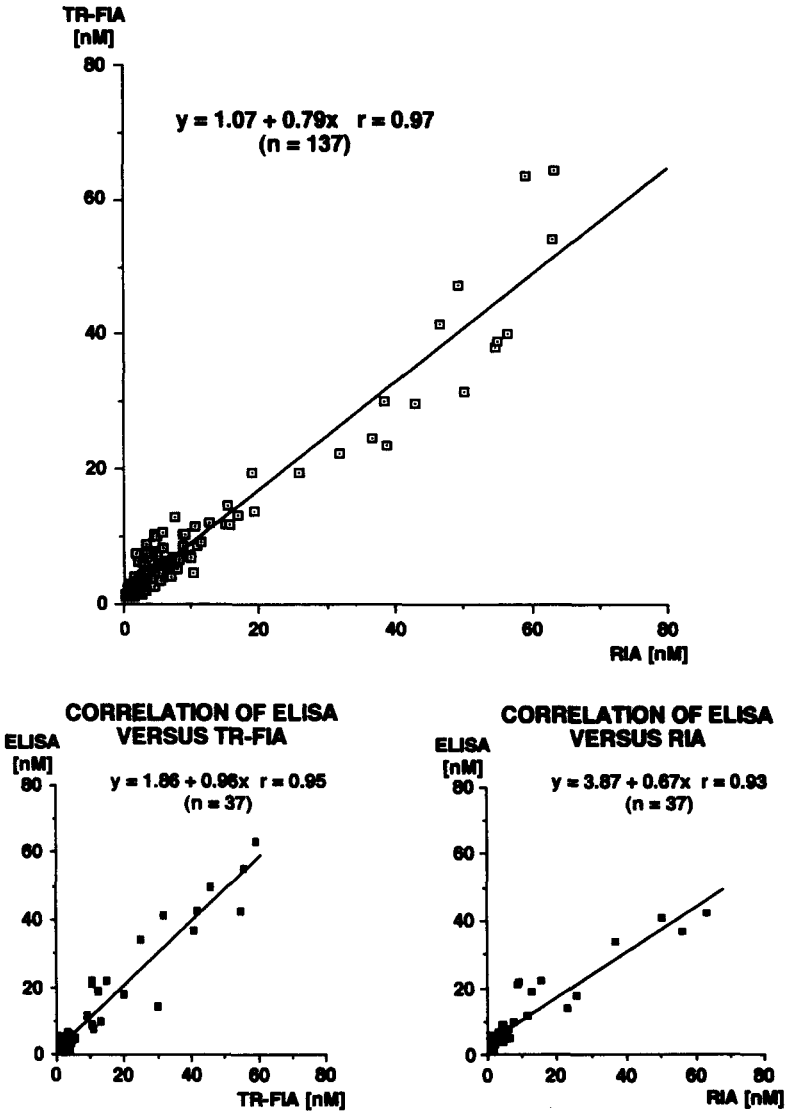


Fig. 6. 137 saliva samples were analyzed in both the commercial RIA adapted for saliva and the TR-FIA (upper panel). 37 of these samples were also measured in the ELISA version of the cortisol assay described (lower panel).

binding, calculation of the mean signal and its standard deviation. The sensitivity was defined as the intercept with the displacement curve of mean minus two standard deviations of the repetitive zero standard signal giving a 95% confidence value. This intercept was found to correspond to a 0.43 nM concentration for a 50 $\mu$l sample. A typical displacement curve and standard curve data are shown in Fig. 5.

*Reproducibility*

The intra-assay variation for saliva cortisol concentrations of 2.2, 5.5 and 13.2 nM ($n = 37$) were 6.7, 4.7 and 4.0%. The inter-assay coefficients of variation for the samples were 9.0, 8.6 and 7.1%, respectively ($n = 50$).

*Correlation of salivary cortisol TR-FIA vs RIA*

One hundred and thirty-seven salivary samples (concentration range: 0.5–63.4 nM) obtained during circadian sampling of healthy volunteers or during exercise-induced stress were analyzed in both the commercial RIA adapted for salivary cortisol measurement [18] and the TR-FIA. Thirty-seven of these samples were also measured in the colorimetric end point version of the cortisol assay (ELISA) described here. The correlation found was very satisfactory ($r = 0.97$) between TR-FIA and RIA with the slope of the regression line 0.79 and the bias + 1.07 nM. The colorimetric assay and TR-FIA showed a correlation of $r = 0.95$, a slope of 0.96 and a positive bias for the ELISA of

Table 2. Recovery and linearity of diluted saliva samples

| Dilution | Factor | Expected value (nM) | Measured value (nM) | (%) of expected value |
|---|---|---|---|---|
| In 0-Std | 1:1* | — | 72.6 | — |
| | 1:2 | 36.3 | 37.0 | 101.9 |
| | 1:4 | 18.2 | 18.9 | 103.8 |
| | 1:8 | 9.1 | 8.1 | 89.0 |
| | 1:16 | 4.5 | 3.7 | 82.2 |
| | 1:32 | 2.3 | 2.2 | 95.7 |
| In 276-Std | 1:2 | 174.3 | 186.9 | 107.2 |
| Mean percentage measured: | | | | 96.6% |

*Undiluted.

1.86 nM ($n = 37$). The ELISA also showed a positive bias when compared to the RIA (3.87 nM) with the slope of the regression line 0.67 and a regression of $r = 0.93$ ($n = 37$). The results are displayed in Fig. 6.

*Recovery*

A salivary sample of 72.6 nM concentration was serially diluted with zero standard. The measured concentration was compared to the expected concentration and expressed as per cent of expected value. In addition, this sample was diluted 1:2 with a 276 nM standard. The mean recovery was 96.6% indicating the good linearity of diluted samples in the assay. The data are summarized in Table 2.

*Interference with serum-contamination in salivary samples*

Saliva pools A and B were spiked with 0.05 to 2.0% (v/v) of serum 1 containing 516 nM cortisol. The expected cortisol concentrations and the measured cortisol concentrations varied in a range of 86.6 to 102% with a mean of 96.5%.

In order to investigate the influence of steroid binding proteins rather than cortisol content in a contaminating serum, to pool B 0.05 to 2.0% of a dexamethasone suppressed serum were added. Recovery ranged from 98.8 to 103% with a mean of 102%.

These results were in the range of the recovery and linearity studies described above, indicating that plasma contamination of salivary samples up to 2% do not interfere with the measured results except by their cortisol content. As saliva samples judged contaminated with blood by inspection were excluded from analysis and the "normal" plasma contamination of saliva samples is in the order of 0.1% [19], no significant interference is to be expected from this potential source of bias. The detailed results of this experiment are shown in Table 3.

*Unstimulated reference levels*

In 46 apparently healthy volunteers [age $22.4 \pm 4.6$ years (mean $\pm$ SD); range 15–33 years] we measured salivary cortisol concentrations at various time points during the day. The results are given in mean $\pm$ 1 standard deviation: 10.2 nM $\pm$ 5.3 (9 a.m.), 4.5 nM $\pm$ 2.4 (12 a.m.), 4.0 nM $\pm$ 2.7 (3 p.m.), 3.1 nM $\pm$ 2.0 (6 p.m.), 1.6 nM $\pm$ 1.7 (9 p.m.). These results are in good agreement with reference values measured by RIA [9].

## DISCUSSION

We have synthesized a cortisol–biotin conjugate by derivatization of C3-CMO to its NHS ester and then coupling this NHS–ester derivative of cortisol to a commercially available biotin–hydrazide derivative which incorporates an amidocaproyl spacer. This spacer facilitates the binding of the cortisol moiety of the conjugate by a specific antibody and the binding of the ureido ring of biotin by streptavidin without steric interference by the two proteins. The conjugate was therefore suitable as a tracer in an immunoassay for the determination of cortisol. As the calculated molecular weight of the tracer molecule is 728.5 Da, in the competitive binding reaction of the immunoassay the analyte and tracer molecule have comparable size. The synthesis and purification of this steroid–biotin

Table 3. Influence of serum-contamination on salivary cortisol levels

| Amount of serum added (%) | Cortisol expected (nM) | Cortisol measured (nM) | Recovery (%) |
|---|---|---|---|
| *Saliva pool A (cortisol: 5.52 nM) mixed with serum 1 (cortisol: 516 nM)* | | | |
| 2.0 | 15.8 | 16.0 | 101 |
| 1.0 | 10.7 | 10.8 | 101 |
| 0.5 | 8.10 | 7.23 | 89.3 |
| 0.2 | 6.55 | 6.62 | 101 |
| 0.1 | 6.04 | 5.24 | 86.6 |
| 0.05 | 5.78 | 5.88 | 102 |
| Mean recovery: | | | 96.9% |
| *Saliva pool B (cortisol: 47.7 nM) mixed with serum 1 (cortisol: 516 nM)* | | | |
| 2.0 | 58.0 | 58.8 | 101 |
| 1.0 | 52.9 | 49.4 | 93.4 |
| 0.5 | 50.3 | 46.1 | 91.7 |
| 0.2 | 48.7 | 47.2 | 96.9 |
| 0.1 | 48.2 | 44.2 | 91.7 |
| 0.05 | 48.0 | 48.9 | 102 |
| Mean recovery: | | | 96.1% |
| *Saliva pool B (cortisol: 47.7 nM) mixed with dexamethasone suppressed serum 2 (cortisol: 36.4 nM)* | | | |
| 2.0 | 48.4 | 49.7 | 103 |
| 1.0 | 48.1 | 47.5 | 98.8 |
| 0.5 | 47.9 | 48.6 | 101 |
| 0.2 | 47.8 | 49.1 | 103 |
| 0.1 | 47.7 | 48.6 | 102 |
| 0.05 | 47.7 | 48.6 | 102 |
| Mean recovery: | | | 102% |

conjugate is easy to perform and can serve as an example for the coupling of biotin to other steroid molecules.

The cortisol–biotin conjugate was employed as a tracer in a non-isotopic immunoassay for salivary cortisol determination. Salivary cortisol levels reflect the biologically active, non-protein bound fraction of total plasma cortisol [3]. The use of biotin as a primary probe features the advantage of a versatile end point determination, i.e. this tracer molecule can be used in laboratories equipped with ELISA readers, TR-fluorometers, potentially with luminometers or in conventional $\gamma$-counting. For the latter case [$^{125}$I]streptavidin has to be employed as the secondary probe.

The use of biotin as a primary probe implies the necessity for an additional incubation step with labeled streptavidin after the immunoreaction and before signal detection. This disadvantage, however, is outweighed by the longterm stability of the tracer molecule. The conjugate has been stored in our laboratory for 24 months without apparent loss of activity. Furthermore, the use of biotin as a primary probe allows for vigorous standardization in the laboratory as only one reagent carrying the detectable label (streptavidin labeled with Europium in case of TR-fluorometric end point) is required for the measurement of different analytes. As up to 13 Europium atoms can be incorporated into one streptavidin molecule, the use of the biotin–streptavidin system also involves an amplification of the detectable signal. The cortisol–biotin conjugate therefore represents a tracer of high specific activity, allowing for small quantities of tracer and of antigen specific antibody to be used in the immunoassay procedure.

The assay described in this article is easy to perform, takes a total of 1.5 h incubation time and has a lower detection limit (95% confidence interval) of $<0.5$ nM. The assay results agree well with those of an adapted commercial RIA kit [18]. The reproducibility of the method is good, linearity of the results is given as indicated in the serial dilution experiment and artificial contamination of saliva samples with serum up to concentrations of 2% (v/v) does not lead to a bias. As described by Hammond and Langley [19], the normal plasma contamination rate of salivary samples is in the order of 0.1%.

In addition to the assay protocol described here, we have tested the stability of microtiterplates carrying a complex of adsorbed anti-rabbit immunoglobulins and anti-cortisol serum diluted in assay buffer. When sealed with a self-adhesive foil, these plates could be stored in liquid state at 4°C for up to 6 months without apparent loss of binding activity for cortisol and the cortisol–biotin conjugate. When employing this assay protocol, a 30 min incubation of the double antibody complex with sample and cortisol–biotin tracer showed saturation binding, thus reducing the total incubation time to $2 \times 30$ min. Following this protocol, the immunoassay is fast, easy to perform and robust. To date, the assay described above has been used for the analysis of more than 40,000 salivary samples from behavioral- and stress-related studies and has shown excellent stability and ease of performance.

In contrast to enzyme–steroid tracers described previously [2] the biotin–steroid conjugate described here is of a molecular size comparable to the analyzed steroid. Chances of interaction with the antibody in the competitive immunoassay by molecular movement are therefore more similar between the tracer and analyte molecule than by use of protein–steroid tracers. A recent publication also employing the streptavidin–biotin system for a TR-FIA for cortisol measurement [20] describes a streptavidin–cortisol conjugate as tracer molecule and Europium-labeled biotin–protein complexes as secondary probe. As the molecular weight of streptavidin is 58,000 Da, in this assay design the tracer and analyte are also of markedly different size.

In contrast to radioiodinated C3-CMO–tyrosine or C3–histamine, normally employed in $^{125}$I-RIA procedures for cortisol determination, the cortisol–biotin conjugate described here has shown intense binding to cortisol-binding globulin (CBG) in serum samples. Ninety-five per cent of the tracer was not bound by the antibody when a serum sample of 50 $\mu$l was introduced into the assay without additional CBG-blocking agents. This percentage of binding reflects the rate of cortisol bound to plasma proteins in circulation. 8-Anilino-1-naphthelene sulfonic acid in a final concentration of 1% (w/v) was not capable of displacing significant amounts of the tracer from CBG, while [$^{125}$I]cortisol was displaced almost completely. With respect to this binding behavior, the tracer molecule described in this article appears to be more similar to unaltered cortisol than [$^{125}$I]tyrosine cortisol tracers in RIAs. Nonetheless, plasma contaminations of the salivary samples in relative concentrations exceeding the

"normal" plasma content of saliva samples by a factor of 20 show no interference due to steroid binding protein effects. Due to its high similarity to unconjugated cortisol in its binding behavior, the conjugate may therefore also prove useful in receptor imaging studies or immunohistochemistry.

*Acknowledgements*—The skilful secretarial assistance of Ms R. Solymar is gratefully acknowledged. The authors wish to thank Ms S. Bauer and Ms K. Schriever for expert technical assistance.

## REFERENCES

1. Murphy B. P., Engelberg W. and Pattee C. J.: Simple method for the determination of plasma corticoids. *J. Clin. Endocr. Metab.* **23** (1963) 293–300.
2. Hubl W., Taubert H., Freimann E., Meissner D., Stahl F. and Doerner G.: A sensitive direct enzyme immunoassay for cortisol in plasma and saliva. *Exp. Clin. Endocr.* **84** (1984) 63–70.
3. Riad-Fahmy D., Read G. F., Walker R. F. and Griffiths K.: Steroids in saliva for assessing endocrine functions. *Endocrine Rev.* **3** (1982) 367–395.
4. Katz F. H. and Shannon I. L.: Adrenal corticosteroids in submaxillary fluid. *J. Dent. Res.* (1969) 448–451.
5. Walker R. F., Riad-Fahmy D. and Read G. F.: Adrenal status assessed by direct radioimmunoassay of cortisol in whole saliva or amniotic fluid. *Clin. Chem.* **24** (1978) 1460–1463.
6. Evans P. J., Peters J. R., Dyhs J., Walker R. F., Riad-Fahmy D. and Hall R.: Salivary cortisol levels in true and apparent hypercortisolism. *Clin. Endocr.* **20** (1984) 709–715.
7. Moore C. A., Stalla G. K. and Müller O. A.: Value of a saliva cortisol radioimmunoassay in the testing of the hypothalamic–pituitary–adrenal axis. *Fresenius Z. Analyt. Chem.* **324** (1986) 260.
8. Rose R. M. and Hurst M. W.: Plasma cortisol and human growth hormone responses to intravenous catheterisation. *J. Hum. Stress* **1** (1975) 22–36.
9. Kirschbaum C. and Hellhammer D. H.: Salivary cortisol in psychobiological research: an overview. *Neuropsychobiology* **22** (1989) 150–169.
10. Wilchek M. and Bayer E. A.: *Methods in Enzymology: Avidin-biotin Technology*. Academic Press, New York, Vol. 184 (1990).
11. Ternynck T. and Avrameas S.: Avidin-biotin system in enzyme immunoassays. In *Methods in Enzymology: Avidin-biotin Technology* (Edited by M. Wilchek and E. A. Bayer). Academic Press, New York, Vol. 184 (1990) pp. 469–481.
12. Strasburger C. J. and Kohen F.: Two-site and competitive chemiluminescent immunoassays. In *Methods in Enzymology: Avidin-biotin Technology* (Edited by M. Wilchek and E. A. Bayer). Academic Press, New York, Vol. 184 (1990) pp. 481–496.
13. Manz B., Heubner A., Köhler I., Grill H.-J. and Pollow K.: Synthesis of biotin-labelled dexamethasone derivatives. *Eur. J. Biochem.* **131** (1983) 333–338.
14. Meyer H. H. D., Sauerwein H. and Mutayoba B. M.: Immunoaffinity chromatography and a biotin-streptavidin amplified enzyme immunoassay for sensitive and specific estimation of estradiol-17$\beta$. *J. Steroid Biochem.* **35** (1990) 263–269.
15. Tiefenauer L. X. and Andres R. Y.: Biotinyl-estradiol derivatives in enzyme immunoassays: structural requirements for optimal antibody binding. *J. Steroid Biochem.* **35** (1990) 633–639.
16. *Amtliche Sammlung von Untersuchungsverfahren nach* §35 *LMBG*, B 82.10, Juni 1985.
17. Soini E.: Pulsed light, time-resolved fluorometric immunoassay. In *Monoclonal Antibodies and New Trends in Immunoassays* (Edited by Ch. A. Bizollon). Elsevier, Amsterdam (1984) pp. 197–208.
18. Kirschbaum C., Strasburger C. J., Jammers W. and Hellhammer D. H.: Cortisol and Behavior: 1. Adaptation of a radioimmunoassay kit for reliable and inexpensive salivary cortisol determination. *Pharmac. Biochem. Behav.* **34** (1989) 747–751.
19. Hammond G. L. and Langley M. S.: Identification and measurement of sex hormone binding globulin (SHBG) and corticosteroid binding globulin (CBG) in human saliva. *Acta Endocr.* **112** (1986) 603–608.
20. Khosravi M. J. and Morton R. C.: Novel application of streptavidin-hapten derivatives as protein-tracer conjugate in competitive-type immunoassays involving biotinylated detection probes. *Clin. Chem.* **37** (1991) 58–63.