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Note

Extraction and quantitation of cortisol by use of high-performance liquid affinity chromatography

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Quantitative determination of cortisol in urine by radioimmunological techniques is inexact and non-specific, probably due to co-determination of other corticosteroids or polar metabolites of cortisol. As a reference method mass spectrometry [1] has been suggested. However, this method is expensive and time-consuming due to the need for derivatization. Low-pressure affinity chromatography has been shown [2] to be useful to enrich cortisol from serum samples prior to high-performance liquid chromatographic (HPLC) quantitation.

A new and faster technique, called high-performance liquid affinity chromatography (HPLAC), has recently been introduced and shown to be useful for enzyme, protein and carbohydrate purification [3, 4]. The technique is based on covalent linkage of a ligand to a solid silica matrix. The ligand, i.e. an antigen or an antibody, has high affinity to the compound of interest and can be absorbed and reversibly eluted with a high ion strength buffer or a compound with high affinity to the substance. In this paper the technique has been used to purify and quantitate cortisol in one step from plasma and urine samples. The extract is directly analysed by HPLC.

EXPERIMENTAL

Materials

Porous silica gel Si 60 (LiChrosorb Si 60, 40 μm) and LiChrosorb RP-18 (5 μm) were obtained from E. Merck, Darmstadt, G.F.R. γ-Glycidoxypropyltrimethoxysilane (Silane Z-6040) was purchased from Dow Chem. (Midland, MI, U.S.A.). Cortisol-3-carboxymethyloxime–bovine serum albumin (BSA)

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was synthesized according to the description of Erlanger et al. [5]. All solvents and other chemicals, obtained from commercial sources, were of analytical grade or HPLC grade and were used without further purification.

Equipment

A Waters Model U6K injector (Waters Assoc. Milford, MA, U.S.A.), a Model 6000 pump and a Model 440 UV detector was used in the chromatographic separations. The LiChrosorb RP-18 column (25 cm × 5 mm I.D.) was eluted and equilibrated with methanol—water (60:40). The flow-rate was set to 1.0 ml/min and the absorbance was monitored at 254 nm. ^{3}H-Radioactivity of collected fractions was measured in Insta-gel (10 ml) on a Packard liquid scintillation spectrometer (Packard, Downers Grove, IL, U.S.A.).

Modification of silica Si 60

Silica Si 60 was coupled to γ-glycidoxypropyltrimethoxysilane according to the method of Glad et al. [4] with minor modifications. A 10-g amount of Si 60 was refluxed with 15 ml of γ-glycidoxypropyltrimethoxysilane for 4 h. A small amount of triethylamine (250 μl) was added. The substituted Si 60 was filtered off and washed with 100 ml of acetone and 50 ml of diethyl ether. Then 2.5 g of the substituted Si 60 were mixed with 3 ml of water, acidified to pH 3 with 0.1 *M* sulphuric acid and heated to 90°C for 1 h. After filtering and washing (10 ml of water, 10 ml of acetone, 10 ml of diethyl ether) the diol compound was oxidized with 25 ml of sodium metaperiodate (0.06 *M* aqueous solution) at ambient temperature for 0.5 h. The substituted Si 60 was then ready for antibody coupling after washing successively with water, acetone and diethyl ether.

Coupling to anti-cortisol antibodies

An antiserum against cortisol-3-carboxymethyloxime—BSA (anticortisol) was raised in rabbits. The antibody solution was fractionated on an ion-exchange DEAE Sepharose Cl 6B column (Pharmacia, Uppsala, Sweden) and eluted with 0.05 *M* Tris, pH 7.4. The immunoglobulin (Ig) fraction was collected. Reamining proteins were eluted with 0.1 *M* NaCl in Tris, pH 7.4, followed by 0.2 *M* NaCl in Tris, pH 7.4. The collected Ig fractions were poured into a dialysis tube and concentrated by means of crystalline PEG 2000. The enriched fraction was dialysed against water. The recovery was 95%, estimated from the absorbance of the fractions at 280 nm. A 4.5-ml volume of anti-cortisol antibody solution dissolved in 10 ml of 0.1 *M* sodium hydrogen carbonate solution was allowed to react with 2.0 g of the modified silica. The mixture was left at 4°C for 22 h with gently stirring. The absorbance at 280 nm of a small portion of the solution was checked intermittently to monitor the coupling efficiency. After coupling, remaining aldehyde functions on the silica matrix were reduced with 80 mg of sodium borohydride, which was suspended in 500 μl of water and added slowly to the solution. The solution was left overnight at room temperature. The material was then filtered off on a G 3 glass filter and washed with 100 ml of 0.1 *M* sodium hydrogen carbonate buffer and 200 ml of distilled water. The yield of the modified silica gel was enough for packing three 5 cm × 5 mm I.D. stainless-steel columns. The columns were packed

with the upward slurry packing technique [6] in water—methanol (30:70) at 14 MPa. The material was stored under water in a refrigerator when not in use.

RESULTS AND DISCUSSION

Test of binding efficiency

The loop of the U6K injector (Waters Assoc.) was replaced by an antibody packed column. A LiChrosorb RP-18 (particle size 5 μm) column was equilibrated with methanol—water (60:40). Ten microlitres of a 10 ng/μl cortisol standard in 0.1 *M* Na_2HPO_4, pH 7.5, buffer were incubated with 10 μl of a [^{3}H]cortisol solution (2.21 μCi/ml) and injected slowly into the antibody column. The antibody column was washed with 1 ml of 0.1 *M* NaH_2PO_4 buffer and 1 ml of water by means of a 2-ml syringe. The cortisol was then eluted with methanol—water (60:40) from the antibody column through the RP-18 column. Fractions of 1.0 ml were collected and counted in a liquid scintillator after the addition of 10 ml of Insta-gel. The data on this and similar experiments show that the ^{3}H-radioactivity was quantitatively (95%) recovered if less than 2 μg of cortisol standard (maximum binding) was injected. Repetitive ion-binding tests after one month's use of the column showed a decrease in binding capacity (20%) but the recovery was unchanged.

Analysis of standard samples

In Fig. 1a is shown a chromatogram of a standard preparation (20 μl of a 1 ng/μl solution). The loop of the injector was replaced by the silica—anti-cortisol column and the knob on the injector was placed in the load position. The injected volume of cortisol solution was 20 μl. After washing the immunosorbent with 1 ml of phosphate buffer and 1 ml of water, the flow direction was changed and the methanol—water (60:40) mixture was introduced to the immunosorbent. A 2-ml volume of this mobile phase was enough to wash out the bound cortisol. The injector was immediately set to the load position again and washed with 2 ml of water. A new injection could be started after washing the antibody column. As seen in the chromatogram (Fig. 1a) cortisol is eluted through the RP-18 column and separated from impurities. Volumes larger than 20 μl could be injected but a tendency to get broad peaks was observed when the volume was 100 μl or more.

Analysis of cortisol in serum and urine samples

A 2-ml volume of serum (or 5 ml of urine) was extracted with 5 ml of dichloromethane. The dichloromethane phase was evaporated to dryness with nitrogen and redissolved in 50 μl of methanol. A 25-μl aliquot was injected onto the antibody column. The column was washed with 2 ml of water. The absorbed fraction was then introduced to the RP-18 column by the procedure given above. The resulting chromatogram (Fig. 1b) shows a cortisol peak which was well separated from minor amounts of impurities. An attempt to analyse cortisol in serum without extraction was also done but after a few injections the RP-18 column became contaminated with impurities probably due to the difficulty in completely washing the antibody column. An external standard

preparation was used to quantitate cortisol because it was difficult to find suitable internal reference compounds which bind to the antibody column.

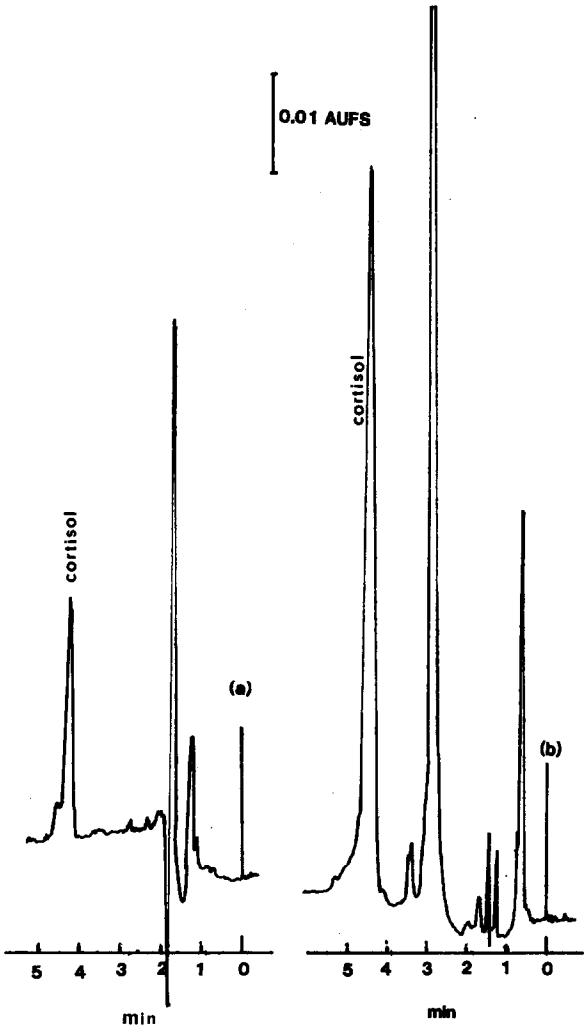

Fig. 1. HPLC chromatogram of (a) a standard preparation (20 ng) and (b) a serum extract. For conditions, see text.

CONCLUSIONS

Enrichment and quantitation of cortisol in one step by use of one affinity chromatography column and one reversed-phase column offer many advantages. One advantage is that a simple non-gradient methanol–water elution system can be used; another is that rather few impurity peaks interfere in the analysis which in turn results in a short analysis time. The anticortisol

column can be used several times and is stable for at least six months if stored in a neutral phosphate buffer (with sodium azide) in a refrigerator. The reported technique can probably find many applications in clinical chemistry laboratories where radioimmunological assays are non-specific.

REFERENCES

1 I. Björkhem, R. Blomstrand, O. Lantto, A. Löf and L. Svensson, Clin. Chim. Acta, 56 (1974) 241.
2 B. Nilsson, L. Tejler and J.-F. Dymling, in G.L. Hawk (Editor), Biological/Biomedical Applications of Liquid Chromatography, Vol. II, Marcel Dekker, New York, Basel, 1979, p. 349.
3 S. Ohlson, L. Hansson, P.-O. Larsson and K. Mosbach, FEBS Lett., 93 (1978) 5.
4 M. Glad, S. Ohlson, L. Hansson, M.-O. Månsson and K. Mosbach, J. Chromatogr., 200 (1980) 254.
5 B.F. Erlanger, F. Borek, S.M. Beiser and S. Liberman, J. Biol. Chem., 228 (1957) 713.
6 P.A. Bristow, P.N. Brittain, C.M. Riley and B.F. Williamson, J. Chromatogr., 131 (1977) 57.