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HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHIC DETERMINATION OF FLUORESCENCE-LABELLED CORTISOL

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SUMMARY

Because of the very low concentration of cortisol in human plasma (6–25 μg per 100 ml), fluorescence labelling is necessary for its *in situ* determination by high-performance thin-layer chromatography (HPTLC). Ready-coated HPTLC plates should be cleaned prior to the application procedure. The fluorescence intensity of cortisol Dns-hydrazone derivatives can be increased by a factor of up to 10 by dipping the HPTLC plates into a liquid paraffin–*n*-hexane solution after development. By this method the detection limit is about 2.5 pg per spot. Quantitative determination of cortisol is possible in the range 10–1000 pg per spot. The serum samples are pretreated by a column extraction. HPTLC opens the possibility of separating several serum samples on one plate simultaneously.

INTRODUCTION

Cortisol in human serum or plasma, an important secretory product of the pituitary function[1], is a parameter for adrenal activity (which is of interest for the diagnosis of, *e.g.*, Cushing syndrome and morbus Addison). Many methods for determining cortisol in biological fluids have been described, including colour reactions[2], UV spectrophotometry[3–5], fluorimetry[6–11], protein-binding methods[12], radioimmunoassay[13–17] and enzymatic reactions[18,19]. These methods are lacking in either sensitivity, reproducibility, specificity or accuracy[10,13,16,17]. On the other hand, chromatographic procedures such as high-performance liquid chromatography (HPLC)[3–5,11,20,21] and thin-layer chromatography (TLC)[19,22,23] give a significant increase in specificity.

Because of the very low concentration of cortisol in plasma or serum (6–25 μg per 100 ml), fluorescence labelling techniques combined with chromatographic separations are now being used with advantage[6,7,9,11].

EXPERIMENTAL

Apparatus

KG-60 HPTLC plates (No. 5631; 10 × 10 cm) were obtained from Merck, and

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used with a Nanoapplicator (Camag, Muttenz, Switzerland), a Nanomat (Camag), a linear developing chamber (Camag) and Hamilton syringes (1000 and 500 μl). Other equipment consisted of a Zeiss KM-3 chromatogram spectrophotometer, a Minigrator (Spectra Physics, Santa Clara, CA, U.S.A.) and CE-1003 Clin-Elut columns (Analytichem Int., CA, U.S.A.).

Reagents

Dns-hydrazine (No. 20197; Serva, Heidelberg, G.F.R.), cortisol (No. 24608), trichloroacetic acid (No. 807), dichloromethane (No. 6050), methanol (No. 6007), benzene (No. 1783), absolute ethanol (No. 972), cyclohexane (No. 9666), liquid paraffin (No. 7174) and dioxane (No. 9671) (all from Merck, Darmstadt, G.F.R.) and butylhydroxytoluene (BHT) (No. 82202; Merck-Schuchardt, Darmstadt, G.F.R.) were used.

Reagent solutions

Dns-hydrazine solution. A 0.02% (w/v) solution of Dns-hydrazine was prepared by dissolving 2 mg of Dns-hydrazine in 10 ml of absolute ethanol and was stored at 4°C in darkness. This solution should be prepared freshly each week.

Trichloroacetic acid solution. A 0.03% (w/v) solution of trichloroacetic acid was prepared by dissolving 30 mg of trichloroacetic acid in 100 ml of absolute ethanol.

Cortisol stock standard solution

A 20-mg amount of cortisol was dissolved in 50 ml of dichloromethane containing 20% of methanol. This solution should be stored at 4°C.

Cortisol working standard solution

The working standard solution was prepared freshly each week by diluting the stock solution to final concentrations of 1500–150 ng/ml.

Determination of cortisol standards

A 1-ml volume of the cortisol working standard solution was evaporated to dryness under a stream of nitrogen and the residue was dissolved in 0.2 ml of trichloroacetic acid solution[6] and 0.1 ml of Dns-hydrazine solution in a 5-ml glass vial. After standing for 30 min at room temperature[11] in the dark, the solution was evaporated to dryness under a stream of nitrogen. The labelled residue was then dissolved in 0.5 ml of absolute ethanol. A 200-nl sample was applied with a Nanoapplicator on to a "pre-washed" HPTLC plate that had previously been at 120°C for 30 min. The HPTLC plate was then developed for a distance of 5 cm in a linear chamber with benzene–dioxane–methanol (8:2:1) containing 0.1% of BHT as the mobile phase. The wet plate was dipped into a mixture of paraffin–*n*-hexane (2:1)[24,25] for 1 sec in order to enhance and stabilize the fluorescence. After drying at room temperature, the plate was scanned directly.

In situ detection

The fluorescence was detected with a chromatogram spectrophotometer at an excitation wavelength of 365 nm (mercury line) and an emission wavelength higher than 460 nm (FL 46 cut-off filter).

Analysis of serum samples

To avoid a time-consuming pre-preparation of the serum[11] and to reduce the sample volume to 2–3 ml of serum, we used a simple extraction method by pipetting 3 ml of serum or plasma directly on to a Clin-Elut ready-for-use column. After addition of 6 ml of dichloromethane and waiting for 3 min, another 6-ml aliquot of dichloromethane was added. The total eluate was collected in a 15-ml glass vial and evaporated to dryness under a stream of nitrogen. The procedure described above was then followed.

RESULTS

The derivatization of cortisol with Dns-hydrazine to form the hydrazone is achieved within 10 min using trichloroacetic acid[6,7]. As the reaction rate was independent of temperature between 0 and 60°C[11], room temperature was used. Derivatization with ethanol–hydrochloric acid as described by Kawasaki *et al.*[11] was not as good as in ethanol–trichloroacetic acid.

As shown in Fig. 1, the hydrazone of cortisol migrates with an R_F value of 0.36 with benzene–dioxane–methanol (8:2:1) as the mobile phase.

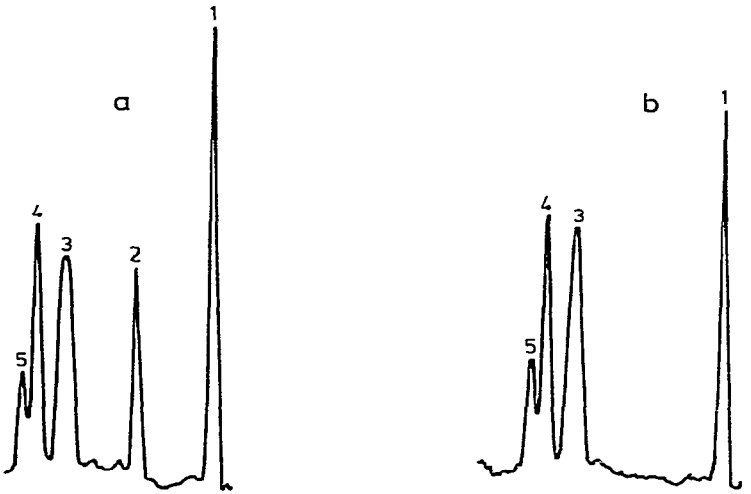

Fig. 1. Chromatogram fluorescence intensity of (a) cortisol Dns-hydrazone and (b) the blank. (1) Origin; (2) cortisol Dns-hydrazone (R_F = 0.36); λ_{ex}, 365 nm (Hg line); λ_{fl}, 460 nm (FL 46); slit width 0.5 mm; scan rate, 50 mm/min; paper advance rate, 60 mm/min; recorder sensitivity, 10 mV.

Fluorescence yield

Dipping of the wet chromatograms[24,25] into a solution of paraffin–*n*-hexane (2:1) directly after development increases the intensity of the fluorescence by a factor of 10 [Fig. 2(a)]. The response remains constant for 1 h and then gradually decreases [Fig. 2(a), unshaded peaks]. If the dipping procedure is repeated after 2 h, the fluorescence yield increases again [Fig. 2(a), unshaded peaks]. When the plate was not pretreated [Fig. 2(a), shaded peaks], the fluorescence reaches only 10% of the possible maximum. If such a plate is dipped for the first time 2 h after development the

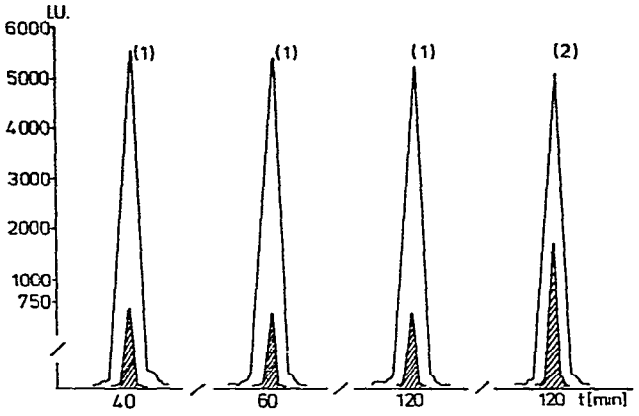

Fig. 2. Effect of paraffin oil–*n*-hexane (2:1) on the fluorescence yield of cortisol Dns-hydrazone. (1) Plate treated immediately after development; shaded peaks, untreated plate; (2) plates treated a second time after 120 min. I.U. = integration units.

fluorescence can be increased [Fig. 2(a), shaded peaks] to 20% of the possible maximum intensity.

The enhanced response of the treated chromatograms is probably due to dissolution of the substance in paraffin oil, which inhibits oxidation or decomposition.

The detection limit for cortisol Dns-hydrazone was about 2.5 pg per spot (Fig. 3).

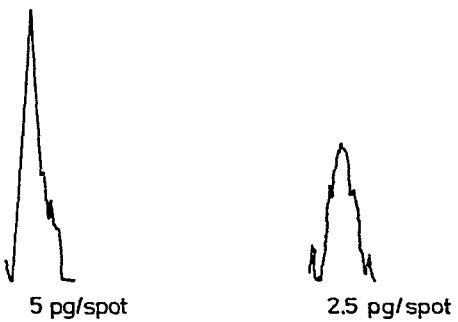

Fig. 3. Detection limit of cortisol Dns-hydrazone. Slit width, 0.5 mm; scan rate, 30 mm/min; paper advance rate, 60 mm/min; recorder sensitivity, 1 mV.

Calibration graphs

The calibration graphs for the cortisol standards are linear in the ranges 10–100 pg (Fig. 4) and 100–1000 pg (Fig. 5) for a 200-nl application volume.

Extraction of cortisol standards

In order to determine the influence of the extraction step with ready-for-use columns, cortisol working standard solutions were applied to Clin-Elut columns. The recovery was 95%. The resulting difference in the chromatographic baselines was negligible (Fig. 6a and b). To evaluate the influence on the extraction of physiological

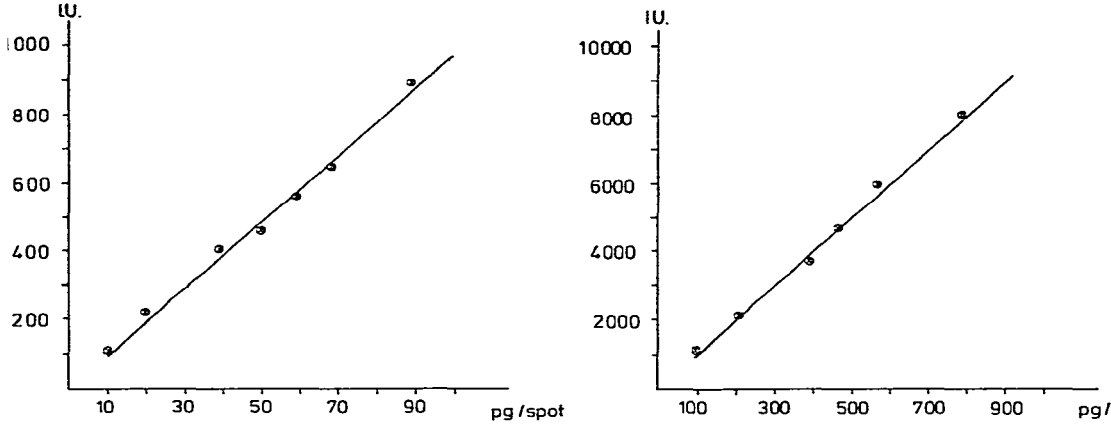

Fig. 4. Determination of cortisol standards as Dns-hydrazones (10–100 pg per spot) by HPTLC. I.U. = integration units.

Fig. 5. Determination of cortisol standards as Dns-hydrazones (100–1000 pg per spot) by HPTLC. I.U. = integration units.

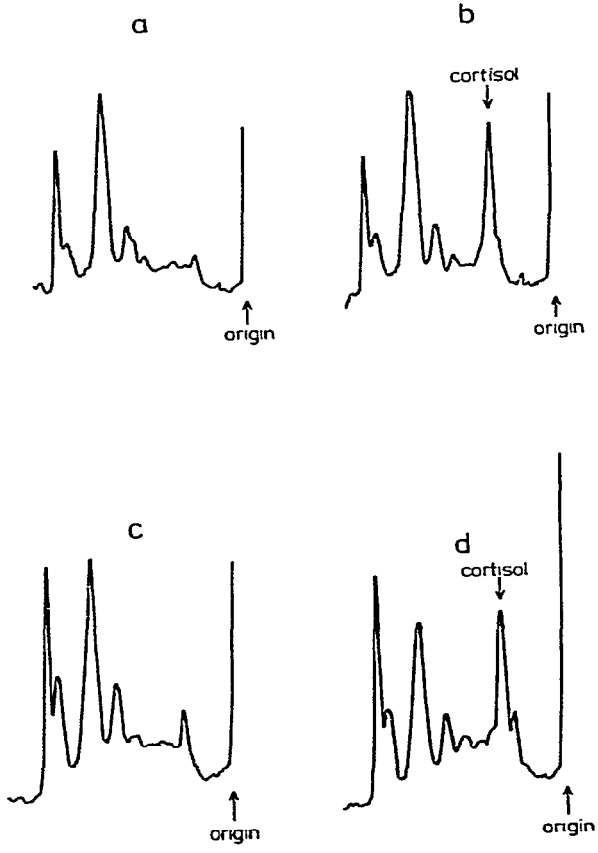

Fig. 6. Local fluorescence intensity after Clin-Elut extraction. (a) Blank; (b) blank spiked with cortisol; (c) blank spiked with albumin; (d) blank spiked with albumin and cortisol.

proteins (serum matrix), bovine serum albumin[3] samples (3% aqueous solution) were spiked with various cortisol working standard solutions. The chromatographic baseline of these samples showed slight differences to those of albumin-free samples, with comparable recoveries (Fig. 6c and d).

Extraction of serum or plasma samples

A normal human serum sample was extracted (Fig. 7a) and the cortisol (Fig. 7b) was found to be 13.2 ± 0.7 μg/100 ml of serum ($n = 9$, coefficient of variation 5.3%); this could be verified by spiking the same serum sample with various amounts of cortisol (Fig. 7c and d). For comparison, the serum of a dexamethasone-treated patient was tested. This sample was similar to a nearly cortisol-free serum, and therefore only a small amount of cortisole was detectable (Fig. 7e).

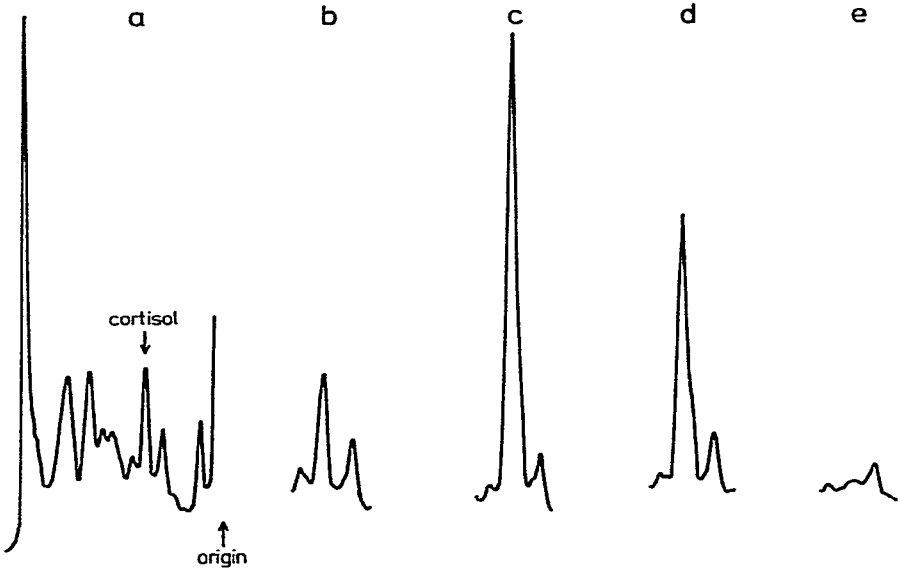

Fig. 7. Local fluorescence intensity of human serum after Clin-Elut extraction. (a) Normal human serum; (b) partial section of (a); (c) partial section of (a) spiked with 480 pg of cortisol; (d) partial section of (a) spiked with 240 pg of cortisol; (e) serum of a dexamethasone-treated patient.

DISCUSSION

The HPTLC determination of fluorescence-labelled cortisol proved to be a specific and highly sensitive method. Using a simple extraction step with Clin-Elut ready-for-use columns, this chromatographic technique is applicable to the determination of cortisol in serum or plasma.

HPTLC is a more practicable, inexpensive and less time consuming method than other techniques[17,18], allowing the analysis of up to 80 samples on one plate at the same time.

Further investigations aimed at spotting even larger sample volumes on the plate are in progress. This should allow the determination of small amounts of cortisol in serum as are found in dexamethasone-treated patients.

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