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Chromatographic Determination of Some Corticosteroids, with Special Reference to Horse Doping *

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Summary. Some chromatographic procedures, which can be used to detect and determine certain corticosteroids in samples from race horses, are described. These procedures include thin-layer, gas and high pressure liquid chromatography.

Zusammenfassung. Es werden einige chromatographische Prozesse beschrieben, die für die Entdeckung des „Doping" von Rennpferden mit gewissen Corticosteroiden geeignet sind. Diese Methoden schließen Dünnschicht- und Gas-Chromatographie, sowie auch die Hochdruck-Flüssigkeits-Chromatographie ein.

Key words. Corticosteroids, chromatographic-determination – Doping – Chromatographic-determination, corticosteroids

Introduction

Horse doping is defined as the administration of certain substances to a horse before a race if these substances can alter the horse's performance on the track. Thus, corticosteroids such as cortisol and its synthetic analogs are as a rule forbidden in connection with horse races (in contrast to the state of affairs in athletics).

Upon arrival at the laboratory, samples from race horses are subjected to screening for the presence of various doping agents. In the case of the corticosteroids, the analytical problems are complicated. Very little work has been reported in the literature about the analysis of corticosteroids in biological material, except for rather unspecific procedures for the determination of cortisol in blood and a few investigations about cortisol metabolites in urine. This is especially the case with the synthetic analogs, where the analytical procedures have been limited to radioactive tracer methods. However, radioimmunoassay methodology for some of these compounds have lately been worked out.

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Pretreatment of the Samples

Since the concentrations of corticosteroids, especially those of the synthetic ones, in body fluids are small, one or more purification steps preceeding the chromatography are necessary. The practice in our laboratory is to first extracting a portion of blood plasma or urine with 10 volumes of carbon tetrachloride. The organic layer, containing many lipid impurities, including more apolar steriod such as progesterone is discarded. Cortisol and its analogs are then extracted with 5 volumes of dichloromethane, and from this extract ionic compounds are removed by shaking the extract with 1/10 volume each of 1N NaOH, 0.1N H_2SO_4 and water respectively. After concentration to a small volume the extract is ready for chromatography.

Paper Chromatography

Although good paper chromatographic systems for the separation of various natural steroids were employed already at 1952 by Bush [1], they have not been used in our laboratory because these procedures are slow and require much work.

Thin-Layer Chromatography

The advantages of this method are simple technology and cheap equipment, and the time required for separation is rather short. In order to further shorten the separation times and to increase the sensitivity, small plates are generally used. Either ordinary microscope slides are coated with adsorbent, or precoated commercial aluminium sheets are cut into smaller pieces.

Most thin-layer chromatographic systems do not have sufficient resolving power. For some applications, it seemed important to discriminate between cortisol and prednisolone, which differ only by one double bond. Such a separation is illustrated in Fig. 1. Besides the reference standard an extract is run. The areas from the sample with corresponding R_f as the standards are marked a and b and can be scraped out, eluted with methanol and subjected to further investigation. The spots can be made visible by spraying with a freshly prepared solution made by mixing equal parts of 0.1 % tetrazolium blue in methanol and 16 % methanolic KOH.

High-Pressure Liquid Chromatography

Ordinary column chromatography with the mobile phase running through the column by hydrostatic pressure is a very slow process. By contrast, high pressure liquid chromatography (HPLC) has many potentialities for detection and determination of small amounts of corticosteroids. A high separating power can be obtained, and with narrow-bore columns and sensitive detectors much smaller amounts can be determined than by thin-layer chromatography. Of great importance for the separating power is the use of a column filling consisting of very small particles with a narrow size distribution. However, this leads to a big resistance to the solvent flow in the column, and therefore a pump, capable of delivering high pressure, is needed to force the mobile phase through the column. A small single-piston reciprocating pump was used in our experiments, capable of delivering pressures of up to 1000 p.s.i. The pulses of the solvent stream are partly eliminated by the elasticy of the Bourdon type pressure indicator

and by incorporating a long piece of capillary tubing in the solvent line. A single-wavelength (254 nm) UV-detector (Chromatronix, Berkeley, Calif., U.S.A.) was used. Fig. 2 shows two separations of corticosteroids. The use of another systems is shown in Fig. 3. While Fig. 2 illustrates a reversed phase system, Fig. 3 shows a straight-phase liquid-liquid partition system, and is a modification of Hesse et al. [2].

Gas Chromatography

Most corticosteroids are not stable to gas chromatographic analysis. However, in recent years procedures for the conversion of the corticosteroids into heat-stable and rather volatile derivatives have been developed [3]. Most methods involve the treatment of the sample with methylhydroxylamine, which reacts with keto groups, followed by reaction with silylating agents to convert hydroxy groups into trimethylsilyl ethers. Elevated temperatures and rather long reaction times are required, especially for dexamethasone [4]. Experiments in our laboratory have shown that relatively large amounts of steroid is necessary to obtain a useful chromatogram, and that interfering by-products are often formed.

A different method, with a basic catalyst, can also be used for silylating certain keto groups. The reaction is faster and only one reaction step is necessary. The catalyst most commonly used is potassium acetate. Fig. 4 shows a gas chromatogram of dexamethasone derivatized with bis-(trimethylsilyl)-triluoroacetamide (BSTFA) catalyzed by potassium acetate (KOAc) at 60° [5] (to 5 μg steroid and 2 mg KOAc 25 μl BSTFA was added. The solution was maintained at 60° for 1h, the solvent was evaporated off under a stream of nitrogen and the residue was dissolved in 25 μl hexamethyldisilazane). As can be seen from the recording, there is one minor peak eluting later – obviously two derivatives are formed. Formation of the minor component could possibly be supressed by changing the reaction conditions.

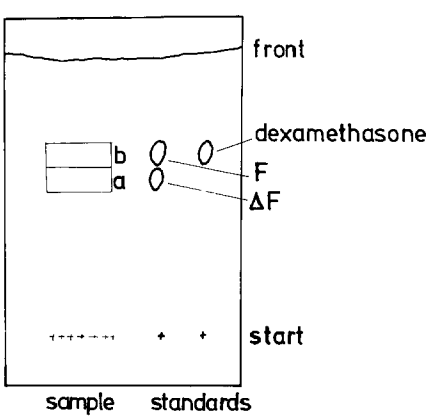

Fig. 1. Thin-layer chromatogram of reference substances (F=cortisol, Δ F=prednisolone, and of a sample extract, corresponding to 1 ml blood plasma, on a TLC aluminium sheet precoated with silica gel 60 F_{254} (Merck), cut into a 10 x 6 cm piece. The mobile phase is the lower layer of the mixture dichloromethane-dioxane-water (2 + 2 + 1) (modification of Hall [6]. The reference markers appear as dark spots in ultraviolet light (254 nm). 1 μg of each steroid is applied

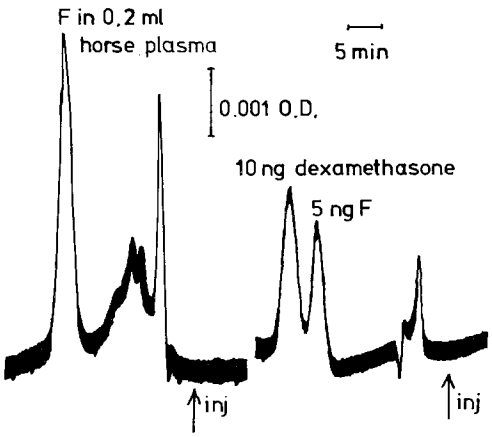

Fig. 2. Right: HPLC of reference substances in 1 μl (F=cortisol). Left: HPLC of extract of horse plasma (5 μl extract). The column is of glass, 50 cm x 1 mm I.D., filled with octadecyl-trichlorosilane treated Porasil T (15 – 25 μ, Waters). Mobile phase: 50 % aqueous methanol, 0.1 ml/min (pressure about 600 p.s.i.). The time axis runs from right to left

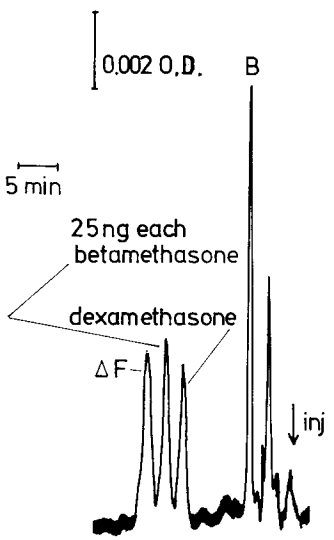

Fig. 3. HPLC of reference compounds in 5 μl (B=corticosterone, Δ F= prednisolone). Column 50 cm x 1 mm I.D. filled with untreated Porasil T (15 – 25 μ, Waters). Mobile phase: chloroform-ethanol-water (948 + 35 + 17, lower layer), 0.1 ml/min

Conclusions

Chromatography is a valuable, almost always necessary technique for the detection, determination and identification of steroids. However, prepurification steps are often necessary, especially when biological material is analysed. Thin-layer chromatography

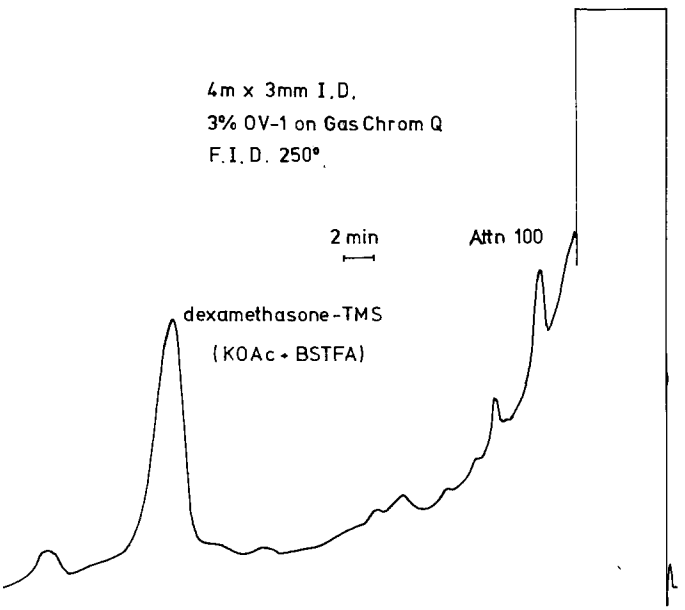

Fig. 4. GC of the trimethyl-silyl derivative of dexamethasone. For the preparation of the derivative, see the text. The conditions for the chromatography are indicated on the fig. (F.I.D.=flame ionization detector). The flow rate of the carrier gas (nitrogen) is 30 ml/min. The injected volume is 5 µl, corresponding to 1 µg dexamethasone

is cheap and easy to perform, but, as a rule, no direct quantitation can be obtained (sometimes spectrodensitometric methods can be carried out, but these require expensive equipment). Gas chromatography (GC) or HPLC give quantitative values directly. HPLC seems to-day to give the most sensitive measurements. For identification, which is most important in the case of doping analysis, the result from a chromatographic run is insufficient. For that purpose chromatography in several different systems is necessary, combined with e.g. color rections on thin-layer plates. However, this is often impossible due to the very small amounts of corticosteroids in the examined sample. A better way is analysis with the combination GC-mass spectrometry [4] which has not been employed in our laboratory due to the lack of such equipment. A further improvement would be the combination of HPLC and mass spectrometry, but no suitable interface for that combination seems yet to exist.

Another procedure, which is going to be investigated in our laboratory, involves combining the results from a chromatographic investigation with those of a radioimmunoassay.

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