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Detection and identification of trace steroids in prednisolone

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Prednisolone samples from different commercial sources produced during 1965–1970 were examined by thin-layer chromatography on silica gel in the system chloroform–methanol–water (180:15:1). When large quantities were applied on the plates several trace compounds were detected. These were eluted from the silica gel and identified by mass spectrometry. The presence of 20-dihydroprednisolone, hydrocortisone, prednisone, 11-desoxyprednisolone, 21-desoxyprednisolone, and prednisolone 21-acetate was detected. The Δ^5-isomer of prednisolone was also found, but this product probably is an artifact formed during the isolation procedure. The different impurities also could be detected in prednisolone after chromatography in the system dichloromethane–dioxan–water (2:1:1). Impurities found in the British Chemical Reference Standard Prednisolone were hydrocortisone (very weak), prednisone and an unidentified product present at the start.

The British Pharmacopoeia for 1968 describes a thin-layer chromatographic method for the detection of foreign steroids in prednisolone and requires that the spots of the impurities should be less intense than those obtained with prednisone and cortisone acetate, applied at a concentration 2% of that of prednisolone. In the U.S.P. XVII, the test for impurities in prednisolone is based upon paper chromatography, and the reference substances, which are cortisone and hydrocortisone, are applied at a concentration of 1% of that of prednisolone. There is no clear indication in these compendia about the structure of the foreign steroids that might be expected in prednisolone. These pharmacopoeias require that the secondary spots observed on the chromatogram be less intense than those obtained with the reference foreign steroids, thereby assuming that the intensity of the colour reaction of the impurities with tetrazolium blue will be similar to that obtained with the reference substances.

A recent publication described how several unknown impurities could be detected in prednisolone when large quantities (300 μg) were applied on a thin-layer plate and when a less specific detection method (ultraviolet fluorescence after a sulphuric acid spray) was used (Nonclercq, Laboureur & Atassi, 1970).

We have identified several foreign steroids by mass spectrometry of the products isolated from the thin-layer plate. We have used the solvent systems described in the Steroid Identification Test of the U.S.P. XVII and by Hall (1964). Several other solvent mixtures were described for the thin-layer chromatography of corticosteroids (Vlassak & Willems, 1964; Sonanini & Anker, 1967; Bellomonte, Carelli & others, 1970) but their merit was not investigated.

MATERIALS AND METHODS

Products

Prednisolone samples produced during the period 1965–1970 by Merck (USA),

Organon (The Netherlands), Roussel (France), and Upjohn (USA) were obtained. The year of manufacture is given on the chromatogram; e.g., I/65 refers to a sample of source I produced in 1965.

Samples of 20α- and 20β-dihydrocortisol were supplied by Professor W. Klyne, Steroid Reference Collection, Westfield College, London; Δ^6-hydrocortisone acetate and Δ^7-prednisolone were obtained from Dr. F. Zeelen (Organon) and Dr. R. Bucourt (Roussel-Uclaf).

Thin-layer chromatography

For analytical chromatograms 300 μg of prednisolone, dissolved in chloroform–methanol (9 : 1), was applied on precoated silica gel plates F254 Merck (thickness 0·25 mm) or on plates coated with silica gel GF254 Merck (thickness 0·25 mm). For preparative purposes 10 mg of material was applied as a narrow band on plates (20 × 20 cm) coated with silica gel GF254 (thickness 0·25 mm).

The chromatograms were developed either with solvent mixture (i): chloroform–methanol–water (180 : 15 : 1) (U.S.P. XVII), or with solvent mixture (ii): the lower layer of dichloromethane–dioxan–water, 2 : 1 : 1 (Hall, 1964).

Spots were visualized under ultraviolet light of 254 nm or, for analytical chromatograms, by spraying with a sulphuric acid–ethanol (1 : 1) solution, heating at 100° for about 10 min, and viewing under ultraviolet light of 365 nm.

Mass spectra

The compounds were isolated by elution of the silica gel removed from the plates with a mixture of chloroform–methanol (1 : 1) use of a Pasteur pipette as a small column, and evaporation of the solvent in a vacuum.

The mass spectra of the reference steroids and of the eluted compounds were obtained through the direct introduction lock of an AEI MS-12 spectrometer at temperatures slowly rising from 150 to 180°. The spectra of the eluted fractions were recorded after disappearance of the more volatile background from the oscilloscope screen. The ion source was operated at 70 eV ionizing voltage, 8 kV accelerating voltage, and 500 μA ionizing current.

RESULTS AND DISCUSSION

Identification of the trace steroids

The structure of the different steroids was determined by comparing the mass spectra of the eluted compounds with those of reference substances or by analysing the mass spectra. Hydrocortisone, prednisolone 21-acetate, and prednisone were identified by comparison with authentic samples. The principal characteristics of these spectra are given in Table 1. Fragmentation patterns of some steroids have been described by Budzikiewicz, Djerassi & Williams (1964), Genard, Palem-Vliers & others (1968), and Spiteller-Friedmann & Spiteller (1969).

The structure of 21-desoxyprednisolone and of 20-dihydroprednisolone could be determined by comparison with the fragmentation pattern of 21-desoxycortisol and of 20-dihydrocortisol respectively. It was not possible to indicate the configuration of the 20-hydroxy group of 20-dihydroprednisolone because the mass spectra of 20α- and 20β-dihydrocortisol were similar, but it may be assumed that this compound has a 20β-configuration because microbial dehydrogenation of the 1,2-position of hydro-

cortisone is known to proceed with simultaneous reduction of the C-20 carbonyl group to a 20β-hydroxyl group (Capek, Hanč & Tadra, 1966).

The structure of 11-desoxyprednisolone was inferred from the presence of a molecular ion at m/e 344 and several fragmentation ions.

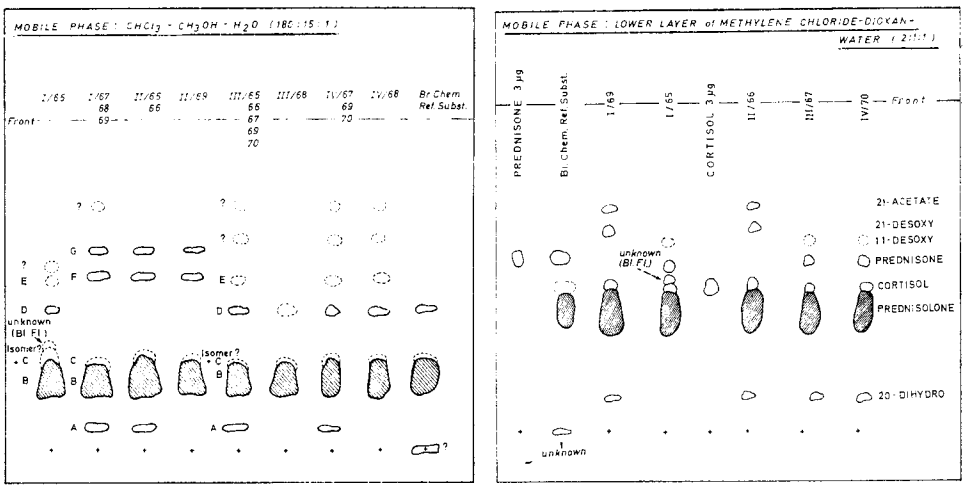
For the separation of hydrocortisone from prednisolone, the product migrating in front of prednisolone in the solvent system (i) was eluted from the thin-layer plate with chloroform–methanol (1 : 1) and chromatographed again. After two developments with solvent mixture (i), two narrow bands appeared moving in front of predni-

Table 1. *Characteristic ions in mass spectra.*

	M^+	M^+-H_2O	$M^+-17\beta$ side chain	$M^+-(17\beta$ side chain $+ H_2O)$	Thermal loss of $(CH_2CO + H_2O)$	Cleavage ring B
Prednisolone*†	360	342	301	283	300	121, 122
Δ^5-Isomer prednisolone† ..	360	342	301	283	300	161
Hydrocortisone*†	362	344	303	285	302	
20-Dihydroprednisolone† ..	362	344	301	283		121, 122
20-α-Dihydrocortisol* ..	364	346	303	285		
20-β-Dihydrocortisol* ..	364	346	303	285		
21-Desoxyprednisolone† ..	344	326	301	283		121, 122
21-Desoxycortisol* ..	346	328	303	285		
11-Desoxyprednisolone† ..	344	326	285	267	284	121, 122
Prednisone*†	358	340	299	281	298	121
Prednisolone 21-acetate*† ..	402	384 342 (-HOAc)	301	283		121, 122

* Reference compound.

† Product isolated from thin-layer plate.



a b

FIG. 1a. Thin-layer chromatogram of prednisolone samples (300 μg) from four sources and of the British Chemical Reference Substance sample on silica gel in the system chloroform–methanol–water, 180:15:1. A: 20-dihydroprednisolone; B: prednisolone; C: hydrocortisone; D: prednisone; E: 11-desoxyprednisolone; F: 21-desoxyprednisolone; G: prednisolone 21-acetate.

b. Thin-layer chromatogram of different prednisolone samples (300 μg) on silica gel in solvent system (ii). For the reference compound (prednisone, cortisol) only 3 μg was applied.

solone. One component was identified as hydrocortisone. The other component was proved by mass spectrometry to be the Δ^5-isomer of prednisolone (11β, 17α, 21-trihydroxypregna-1,5-diene-3,20-dione). This compound appears to be an artifact formed during the isolation procedure. This was demonstrated by comparing the spot intensity of the Δ^5-isomer in the chromatograms of fresh and old (from 4 to 48 h) solutions of prednisolone in chloroform–methanol. Keeping a solution of prednisolone in chloroform–methanol seems to favour a migration of the double bond from the Δ^4- to the Δ^5-position. Possibly some samples actually contain a small amount of the Δ^5-isomer.

Examination of the prednisolone samples

Prednisolone of source I manufactured in 1965 contained hydrocortisone, prednisone, and a very small amount of 11-desoxyprednisolone as impurities. An unknown impurity was present in front of the hydrocortisone spot, which gave a blue fluorescence in the ultraviolet after spraying with sulphuric acid (Fig. 1a). This impurity also could be observed using solvent system (ii) (see Fig. 1b). Later samples from the same source contained 20-dihydroprednisolone, 21-desoxyprednisolone and prednisone 21-acetate.

Prednisolone from source II contained the same impurities as most samples from source I. It should be noted that one sample manufactured in 1969 did not contain 20-dihydroprednisolone. This result is probably accidental, because the same observation was made with one sample from source III and one from source IV. As this impurity is probably formed during the bacterial dehydrogenation at the 1,2-position of hydrocortisone, it may be assumed that the reduction of the 20-keto-group can be negligible during some fermentations.

The foreign steroids in prednisolone samples from sources III and IV are 20-dihydroprednisolone, hydrocortisone, prednisone, and very small amounts of 11-desoxyprednisolone. The impurities found in the products from these two sources were similar.

The British Chemical Reference Substance Prednisolone was examined in solvent system (i). Impurities with R_F values of prednisone and hydrocortisone were detected besides a product remaining at the start. This sample was also chromatographed with solvent mixture (ii), which is mentioned in the report of the panel responsible for the establishment of the Reference Substance. Besides the polar impurity, two spots with R_F values, relative to prednisolone, of 1·14 and 1·30 were found. The impurity with R_F of 1·30 is described in the report accompanying the sample as the 4,5-dihydro-derivative of prednisolone. The mass spectrum of this product, isolated by using solvent system (i) or (ii), identified it as prednisone. The second very weak spot with R_F 1·14 corresponded to hydrocortisone. This compound could not be identified by mass spectrometry after separation with solvent mixture (i) (two developments) or (ii), possibly because of lack of sufficient material. The identification of the compound remaining at the start was not attempted.

Several other samples of prednisolone were also examined with solvent mixture (ii) (Fig. 1b). This system likewise proved to be satisfactory for the analysis of prednisolone, particularly for the detection of hydrocortisone. It was less suitable for the identification of foreign steroids by mass spectrometry because more decomposition was observed in the products isolated from plates developed with this system. The

eventual presence of the Δ^5-isomer of prednisolone in the different samples could not be detected because this impurity coincides with the prednisolone spot.

It was difficult to determine the quantity of the foreign steroids in the prednisolone samples because the corresponding reference substances were not available. Comparison of the intensity of some spots for which pure substances were available, however, indicated that the amount is lower than 1% and generally lower than 0·5%.

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